Revealing the mechanism behind IL-23 driven autoimmunity

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Disclaimer

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- Gyülvészi G, Haak S, Becher B.:
  IL-23-driven encephalo-tropism and Th17 polarization during CNS-inflammation in vivo *Eur J Immunol. 2009 Jul;39(7):1864-9.*

- Haak, S., Gyülveszi, G., Becher, B.:
  Th17 cells in autoimmune disease: changing the verdict *Immunotherapy 1 vol.2 199-203*

- Codarri, L., Gyülveszi G., Becher B.:
  GM-CSF secretion by T_H cells is dependent on RORγt and essential for autoimmune neuro-inflammation *(submitted)*

Summary

The immune system is remarkably effective in protecting the host against pathogenic insults. Despite several layers of self-tolerance mechanisms, the immune system is potentially capable to mount immune responses against self-antigens causing autoimmune diseases such as multiple sclerosis (MS). MS is characterized by the infiltration of inflammatory leukocytes into the central nervous system (CNS) resulting in axonal injury and demylination. During autoimmune neuro-inflammation, helper T (T_H) cells initiate tissue damage and neurological impairment. Initially, IFN-γ-producing T_H1 cells together with IL-12 were thought to be the driving force behind the inflammation observed in MS. Surprisingly however, it was only later found that the deletion of IFN-γ and the T_H1 inducing cytokine IL-12 led to exacerbated disease development in experimental autoimmune encephalomyelitis (EAE), which serves as the animal model for MS. Now, it is widely held that IL-17-secreting T cells (T_H17) rather than T_H1 cells are the main encephalitogenic population in autoimmune inflammation and IL-23 supports the expansion/survival of IL-17-secreting T helper cells. To this day, none of the known T_H17 signature cytokines (IL-17A, IL-17F, IL-22, IL-21) has been shown to be mandatory for the development of EAE. Nevertheless, IL-23 as well as the T_H17 transcription factor RORγt elicits an encephalitogenic program, which leads to the production of a so far unknown encephalitogenic factor or combination of factors and ultimately initiates the development of EAE.

We demonstrated that IL-23-medaited effect on T cell pathogenicity is a feature far beyond T cell polarization. IL-23 not only promotes T_H17 polarization but also and more critically influences T cell CNS-tropism. The lack of IL-23R engagement interferes with the capacity of T cells to produce pro-inflammatory cytokines (IL-17A, IFN-γ and GM-CSF) *in vivo* and to acquire pro-inflammatory properties.

Furthermore, we showed that rather than „conventional“ T_H17 cytokines, GM-CSF production marks the population of neuro-encephalitogenic T cells and its production is dependent on the activity of the IL-12/23 receptor complex and RORγt. Conversely, IFN-γ, IL-12 and IL-27 inhibit RORγt expression and the secretion of GM-CSF.

Taken together, here we describe that IL-23 elicits an encephalitogenic program in neuro-Ag-reactive T cells which leads to their ability to invade the CNS and promotes the production of GM-CSF which, in contrast to IL-17 or IFN-γ, is the only known T cell-derived factor with a non redundant function in CNS autoimmune disease today.

Zusammenfassung

Das Immunsystem ist ausgesprochen effektiv darin, den Wirt gegen eindringende Pathogene zu schützen. Trotz verschiedener Mechanismen die zur Toleranzentwicklung beitragen, kann es unter Umständen zu einer Immunantwort gegen Selbst-Antigene kommen, was zu Autoimmunerkrankungen wie der Multiplen Sklerose (MS) führen kann. MS wird durch das Einwandern von entzündungsinduzierenden Leukozyten ins zentrale Nervensystem (ZNS) ausgelöst. Dies hat axonale Schäden und Demyelinisierung zur Folge. Während dieser autoimmunen, neuronalen Entzündungsreaktion, initiieren Helfer T (T_H) Zellen die Gewebeschädigung und die damit verbundene neurologische Beeinträchtigung. Lange hat man geglaubt, dass in erster Linie IFN-γ-produzierende T_H1 Zellen zusammen mit IL-12 für diese neuronale Entzündung in MS verantwortlich sind. Allerdings wurde später entdeckt, dass die Neutralisierung von IFN-γ sowie von dem T_H1-induzierenden Cytokin, IL-12, zur Verschlimmerung des Krankheitsbildes in der experimentellen autoimmunen Encephalomyelitis (EAE), dem Tiermodell für MS, führt. Heutzutage wird weitgehend davon ausgegangen, dass IL-17A ausschüttende T Zellen (T_H17) anstelle von T_H1 Zellen die Hauptpopulation der encephalitogenen Zellen in der autoimmunen Entzündung darstellen. Diese T_H17 Zellen und deren Expansion/Überleben werden durch das Cytokin IL-23, unterstützt. Bis heute, konnte jedoch keinem der T_H17 spezifischen Cytokine (IL-17A, IL-17F, IL-22, IL-21) eine essentielle Rolle in der Entwicklung von EAE zugeschrieben werden. Nichtsdestotrotz führt IL-23 sowie der T_H17-Transkriptionsfaktor RORgt zu einem encephalitogenen Programm, welches zur Produktion von einem oder einer Kombination von bisher unbekannten encephalitogenen Faktoren führt, welche schlussendlich die Entwicklung von EAE hervorrufen.

Wir konnten zeigen, dass der IL-23-vermittelte Effekt auf die Pathogenizität von T Zellen eine Eigenschaft ist, die weit über die T Zell-Polarisierung hinaus geht. IL-23 begünstigt nicht nur die Polarisierung von T_H17 Zellen, sondern beeinflusst ebenfalls die Migration dieser Zellen ins ZNS. Das Fehlen des IL-23R interferiert mit der Kapazität von T Zellen, pro-inflammatorische Cytokine (IL-17A, IFN-γ und GM-CSF) in vivo zu produzieren und somit pro-inflammatorische Eigenschaften zu erwerben. Weiter haben wir gezeigt, dass GM-CSF Produktion die neuro-encephalitogene T Zell Population besser kennzeichnet als die konventionellen T_H17 Cytokine. Die Produktion von GM-CSF ist außerdem von der Aktivität des IL-12/23 Rezeptor-Komplexes sowie von dem Transkriptionsfaktor RORgt abhängig. Im Gegenzug hemmt IFN-γ, IL-12 und auch IL-27 die RORgt Exprimierung sowie die Ausschüttung von GM-CSF.

Zusammengefasst konnten wir zeigen, dass IL-23 ein encephalitogenes Programm in neuroantigenreaktiven T Zellen hervorruft, welches diese befähigt, ins ZNS einzuwandern. Zusätzlich zeigen wir, dass IL-23 die Produktion von GM-CSF positiv beeinflusst, welches im

Gegenteil zu IL-17 oder IFN-γ, der einzig bekannte von T Zellen stammende Faktor ist, der bis heute eine nicht-redundante Funktion in ZNS-Autoimmunerkrankungen hat.

Immune system

The immune system is a complex network of organs and cells connected by signaling molecules, which main function is to protect the host organism against intracellular and extracellular intruders such as viruses, bacteria, fungi and helminthes. It can be further divided into two highly specialized but cooperating systems: innate and adaptive immune system.

The first line of defense: the innate immune system

Innate immune system is evolutionary more ancient, and can be found in all vertebrates, insects and plants and use a primitive, non-specific recognition system to combat against infectious agents (Yoshimura et al., 2006). It represents the first defense line of the organism against pathogens prior to the activation of the adaptive immune system. It composed of natural killer cells (NK), γδ T cells and phagocytic cells such as monocytes, macrophages, dendritic cells and polymorhonuclear neutrophils. These cells recognize conserved components (DNA, cell wall elements) of microorganisms by the use pathogen-associated molecular pattern receptors (Janeway and Medzhitov, 2002). Engagement of these receptors enhances phagocytic activity and ultimately leads to the destruction of pathogens. Activated innate immune cells release pro-inflammatory cytokines and chemokines in order to orchestrate the subsequent innate immune respond (Beutler, 2004; Medzhitov and Janeway, 1997).

The ability of “remembering”: the adaptive immune system

The adaptive immune system is only present in higher vertebrates and its cells specifically recognize intra- and extracellular pathogens by their specific receptors, which are generated by somatic recombination. Cells in this immune compartment fall into two basic categories, T and B-lymphocytes. B cells combat extracellular pathogens by producing antibodies, they mature in the bone marrow and on the cell surface they bear specific B cell receptor (BCR) that is composed of membrane bound antibody molecules. Antibodies are a heterogeneous mixture of glycoproteins and they differ in size, charge and amino acid composition. In mice and humans, five distinct groups of antibodies can be distinguished, namely, IgG, IgA, IgM, IgD and IgE. The primary function of an antibody is to bind antigen. In some cases this binding has direct effect by neutralizing bacterial toxins, but more often the interaction of antibody and antigen is without

significance unless secondary effector functions are involved such as the complement system (Calame et al., 2003).

T cells are generated in the bone marrow from common precursor cells but subsequently they migrate to the thymus for maturation. In the thymus they undergo positive selection for the recognition of the major self-histocompatibilty complex (MHC) and negative selection. During negative selection, T cells bearing receptors, which recognize self-antigens, are eliminated from the repertoire by apoptosis. T cells are only able to sense processed antigens presented in the context of MHC molecules (Zinkernagel and Doherty, 1974). Cytotoxic $CD8^+$ T cells interact with MHC class I, which is virtually present on the surface of all cells of the body while $CD4^+$ T cells establish interaction with MHC class II on the surface of DCs. For proper T cell activation, TCR and MHC class II interaction together with additional co-stimulatory signals is required and this process is called priming (Quezada et al., 2004). T cells have a wider range of activities than B cells. One subset of T cells provides help to B cells, while others interact with cells of the innate system in order to maximize their phagocytotic capacity or a third subset recognizes virus infected cells and destroys them (Appay et al., 2002).

The adaptive immune system evolved a remarkable capacity, namely the ability of “remembering” which makes the immune respond more efficient and faster. These two, highly specialized arms of the immune system rely on each other and they work in complementation to provide the best protection to the host (Medzhitov and Janeway, 1997, 1998).

Tolerance

Most infections in a healthy individual are short-lived and do not cause permanent damage since our immune system combats the pathogen. Because of the random specificities expressed initially by developing B and T lymphocytes, many of these cells could carry an autoreactive specificity posing the threat of autoimmunity (Gay et al., 1993; Nemazee and Buerki, 1989). To avoid autoimmunity, the immune system has evolved several layers of self-tolerance mechanisms which eliminate or inactivate immune cells that bear receptors specific for autoantigens (Goodnow et al., 2005). During negative and positive selection in the thymus, auto-reactive cells are forced to initiate an apoptotic pathway thus to a larger extent are eliminated from the repertoire. This process is also known as central tolerance.

The immune system has evolved to recognize and eliminate threats coming from the outside, and as well it is well equipped with mechanisms to protect itself against self-destruction. The innate and adaptive effectors mechanisms consist of the production of pro-inflammatory cytokines and chemokines, recruitment and activation of effector cells, which at the end results in the

destruction of infected cells. In an ideal case these mechanisms only recognize and destroy foreign pathogens whilst they ignore the components of our body. Despite the selection mechanisms, self-reactive immune cells can be found in our body and if they are not tightly controlled they pose an immediate threat of autoimmunity. Two fundamental mechanisms play an important role in this process; first, the elimination of self-reactive cells in the thymus during development and later on, generation of peripheral regulatory cells (Kronenberg and Rudensky, 2005; Lohr et al., 2005; Tang and Bluestone, 2006).

Because many of the proteins which the body is composed of are not expressed in thymus or serum, and in some cases not expressed until after the immune system has matured there need to be some mechanisms to prevent autoreactivity of lymphocytes after they have emigrated from the thymus/bone marrow. These mechanisms are defined as the peripheral tolerance.

The first layer of peripheral tolerance is ignorance. Autoreactive T and B-lymphocytes ignore the presence of their autoantigen because either it is presented in too low concentrations or expressed in such sites – eye, testis, central nervous system- that are not freely exposed to immune surveillance. The second mechanism is anergy, which means that naive T cells do not become activated without co-stimulatory signals even if their autoantigen is presented in the context of MHC molecules. The third component of peripheral tolerance is the phenomenon termed dominant regulation or suppression where autoagressive T lymphocytes are prevented from reacting by the presence of a special class of regulatory T cells.

Autoimmunity

Despite these strict tolerance mechanisms autoimmunity can develop and result in severe disease. Autoimmune diseases affect approximately 5% of the world population and compromise a heterogeneous group of insufficiently understood disorders. Some of these diseases are systemic such as systemic lupus erythematosus, some are tissue specific such as multiple sclerosis (MS) and rheumatoid arthritis (RA). The cause of most autoimmune diseases is unknown but most seems to arise out of a combination of a genetic susceptibility (loss of tolerance to self-antigens; abnormally activated T helper cells) and environmental factors (infection, diet, toxic environmental components) (Rioux and Abbas, 2005). It is becoming increasingly clear that the different immune cell types involved in autoimmunity communicate with one another. As key elements of this communication network, cytokines and chemokines orchestrate the recruitment, survival, expansion, effector function and contraction of autoreactive lymphocytes in autoimmunity. The traditional view of the role of cytokines -pro-inflammatory cytokines promote inflammation meanwhile regulatory cytokines inhibit cell-mediated immune

responses- in autoimmunity was simple to explain the complex mechanisms underlying autoimmunity. Cytokines work together as a complex network and as a result, their actions have a central role in the development of autoimmunity and autoimmune disease (O'Shea et al., 2002).

Multiple sclerosis

The first proper case report of Multiple Sclerosis (MS) was in the early 19th century described by Jean-Martin Charcot. Nowadays, MS is the most common chronic inflammatory disease of the central nervous system (CNS) affecting mostly young individuals and the number of afflicted people worldwide is around a million individuals (Sospedra and Martin, 2005). Unfortunately, over time the disease develops into severe motor disability in more than half of them. The common pathological hallmarks of MS include CNS perivascular infiltration of inflammatory cells, resulting in plaques of demyelination, astrogliosis, axonal injury and ultimately neurologic deficit. Typical clinical symptoms of MS are the following: weakness of extremities, local sensory losses and visual impairment (Owens et al., 2001). Two mayor forms of MS can be distinguished based on their clinical and pathological differences. In the relapsing-remitting (RR-MS) form, which affects twice as many women as men, the disease often starts with an immunological attack which lasts from a few days to weeks and this attack is followed by either a partial or a complete recovery. The symptom-free period might last from a few months to years. The RR phase can last from five to ten years, and after this period, the disease evolves into the chronic-progressive stage. In this stage, distinct attacks are rare and remissions tend to disappear and CNS tissue seems to be permanently damaged. Very often people suffering of this form of MS are unable to walk which leaves them wheel chair bound for life. The second mayor form of MS is called the primary-progressive MS and affects about 10-15 % of patients. This form is characterized by the complete lack of distinct inflammatory attacks; it has a slow steady onset, very often starting with walking difficulties that are followed by worsening motor dysfunctions which might result in complete leg paralysis after years (Steinman, 2001; Teunissen et al., 2005).

The etiology and pathogenesis of multiple sclerosis

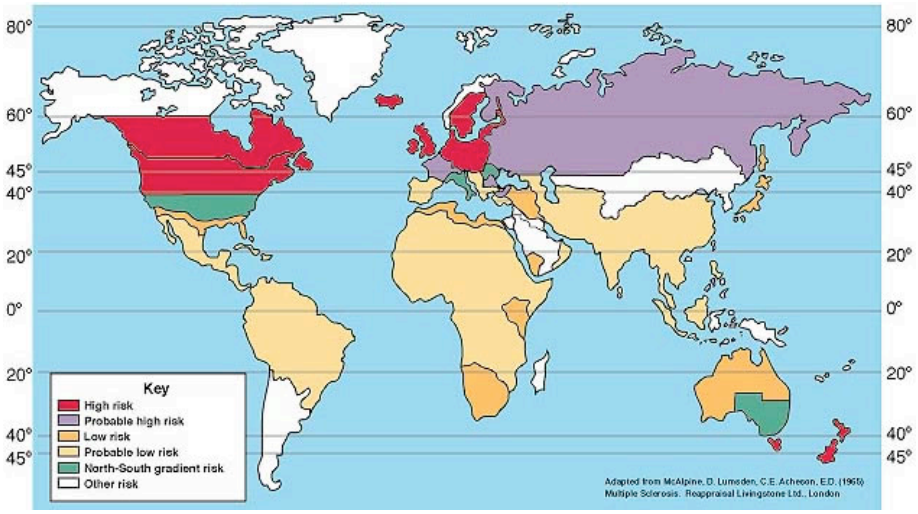

Figure 1: World distribution of multiple sclerosis

The etiology of MS remains unclear but innumerous studies suggest that both genetic factors and environmental triggers contribute to disease development. Interestingly, the prevalence for MS varies between certain geographic locations. North America and northern Europe are considered, as a high-risk area with a 1 in 500 probability while in Japan the prevalence is 10-fold lower in the general population. It has been shown that the risk of developing MS significantly increases among those people who have a relative afflicted with MS. For example first-degree relatives of MS patients have a 20- to 50-fold higher risk to develop the disease (Dyment et al., 2004; Martin et al., 1992). This observation strongly supports the idea that genetic factors are involved in the development of the disease. In the last decades hundreds of studies investigated the contribution of different genes to MS. It was found that one of the mayor contributors to MS can be found in the proximity of the mayor histocompatibilty complex (MHC or HLA) on chromosome 6 region 21. This region is thought to account for 10%-60% of the genetic risk of MS (Haines et al., 1998).

Other genes within the HLA complex are pointed out as possible risk factors of MS as well but certain connection between the polymorphism of a gene and the development of MS is hard to be proven. Studies investigating the role of different genes differ in methodologies and sample size, ethnic background of patients and patients are not always normalized with respect to HLA, clinical or pathological phenotype. The following genes are thought to be implicated in the development of MS: TGF-β family members, IL-1, estrogen receptor, CTLA-4 and TNF-α while the polymorphism of these genes might confer protective effects: CCR2 and IL-10Rα (Haines et al., 2002).

Among non-genetic factors, differences in behavioral/lifestyle and infectious agents have been proposed to induce or contribute to MS development. It is an interesting observation that female patients outnumber men, which might suggest that hormonal differences could contribute to

disease development. This observation is supported by the following facts: amelioration of disease during pregnancy, menstrual cycles coincide with relapses and estriol has therapeutic effects in MS. The exact mode of action of sex hormones remains elusive but certain studies suggest the influence of estrogen on the secretion on pro-inflammatory molecules while male hormones might exert an opposite effect (Runmarker and Andersen, 1995; Sicotte et al., 2002).

It has been shown in studies that the prevalence of MS decreases from north to south, which might be in relation with the increased exposure to UV light. Several explanations have been offered in the last several years why this phenomenon could happen. UV light is an important inducer of vitamin D synthesis, which has been proved in animal experiments to exert beneficial effects (Hayes, 2000; Spach and Hayes, 2005). Another hormone, which is regulated by sunlight, is melatonin and in the absence of sunlight, the excess of melatonin could induce the activation of inflammatory T cells resulting in uncontrolled inflammation (Hayes, 2000).

Viral and bacterial infectious agents are fashionable and would be straightforward candidates as the cause of MS. Many studies have claimed to identify the infectious trigger of MS but none of them could show the direct and exclusive link between the infectious agent and disease development (Sibley et al., 1985). It is well known that viral infections are very often followed by relapses. The DNA of some human pathogens encodes for conserved protein motifs, which can be found in our body too for example in the myelin sheath. Our immune system recognizing this foreign protein not only tries to fight against the invading infectious agent but might mistakenly attack our self-protein too, leading to autoimmunity in this case to MS. This process is often referred to as molecular mimicry (Croxford et al., 2005). Genes that encode sequences that mimic mayor structural proteins of the myelin sheath can be found in several viruses such as Epstein-Barr virus, measles virus, herpes virus 6 and papilloma virus. Although we have several infectious candidate agents as a cause of MS, it seems that the Koch paradigm (one disease, one infectious agent) cannot be applied to MS. It is suggested by all these studies that most likely MS develops on a susceptible complex genetic background in concert with certain environmental and infectious factors.

Pathogenesis of multiple sclerosis

It is thought that autoimmune dysregulation is responsible for the immune attack against the CNS. As discussed previously, the basic concept proposes that exposure to environmental pathogens together with predisposed genetic background leads to activation of auto-reactive T cells that recognize central nervous system auto-antigens, leading to inflammation and demyelination. Support for the view that MS is an immune-mediated disease has been based

largely on the presence of activated myelin-reactive T-cells; inflammatory infiltrates of $CD4^+$, $CD8^+$, and γδ T-cell receptor (TCR)+ T cells, plasma cells, macrophages and DCs in the lesions (Chavarria and Alcocer-Varela, 2004; Ewing and Bernard, 1998). Vaccination of susceptible animals with myelin basic protein results in a relapsing-remitting, inflammatory, demyelinating CNS disease called experimental autoimmune encephalomyelitis (EAE), which is used as an animal model of MS (Slavin et al., 1998). The role of an autoimmune response in this model was confirmed by adoptive transfer experiments, which demonstrated that $CD4^+$ T cells from diseased animals can transmit the disease to naive animals (Sospedra and Martin, 2005).

Treatment for multiple sclerosis

In order to influence the clinical outcome of MS, the most widely used approach is disease modifying drug therapy. For the treatment of RR-MS there are six approved drugs available on the market. Three of them are different types of Interferon-β; originally described as an antiviral agent, it is currently the most frequently used medication for MS. Only in the recent years it has been shown that it has immunomodulatory activities. Its mode of action is diverse, including the up-regulation of the production of anti-inflammatory cytokines such as IL-10, blocking the entrance of lymphocytes into the central nervous system (reduces blood brain barrier (BBB) opening almost immediately after administration) and preventing the adhesion of lymphocytes to the cells of the BBB. Unfortunately IFN-β only reduces the disease severity by 30% and does not have a major impact on disease progression (Kieseier et al., 2008; Weinstock-Guttman et al., 2008).

Another approved drug is glatiramer acetate (GA) that does not have superior affects on MS than interferon-β but its side affect's profile is more favorable. Interestingly GA was developed to mimic a certain protein in the myelin sheath and to induce EAE but it turned out that instead of causing disease it blocks efficiently disease onset. Its mode of action is not yet fully understood but most likely it interferes with the presentation of autoantigenic peptides by antigen presenting cells and it directs T cell polarization towards T_H2 responses. These T cells and their cytokines are supposed to be beneficial in MS by ameliorating disease development. (Johnson et al., 2003; Teitelbaum et al., 2004)

Another promising therapy for MS treatment is the use of monoclonal blocking antibodies against VLA-4 (Nataluzimab), IL-2 receptor (Dacluzimab) or IL-12p40 (Ustekinumab). The first one effectively blocks the migration of auto-aggressive T cells into the CNS and reduces brain inflammation (Miller et al., 2003), while the use of Dacluzimab effectively stimulates and expands immuno-regulatory NK cells and impairs T cell activation and further clonal expansion. Compared to IFN-β, monoclonal antibodies are more efficient; they are able to reduce inflammation under

certain circumstances by 80% and proved to be successful where IFN-β treatment fails (Coles et al., 1999). The last one blocks the action of both IL-12 and IL-23. The presence of IL-23 in animal models seems to be vital for the development of organ-specific inflammation but in human clinical trials the use of the blocking antibody failed to exert beneficial effects on MS progression (Elliott et al., 2009; Ryan et al.; Segal et al., 2008).

The application of these drugs in MS treatment is certainly an enormous improvement on MS patients' life quality and the list of possible drug and drug targets could be continued long such as chemokine receptor inhibitors, CD4 blocking monoclonal antibodies, DNA vaccination and numerous others. Many of these targets come from animal experiment studies where they appeared to be promising but later did not show activity in MS. Therefore caution must be taken in extrapolating animal data to humans (Conway and Cohen).

But is there a complete and definitive cure for MS? Early studies showed no renewal potential in adult CNS but recently these observations are disproved and new hopes have been raised by the preliminary results obtained from transplanting stem cells into demyelinated rodents. Spontaneous re-myelination happens in the adult brain under diseased conditions too but this process fails over time in MS. The use of stem cells provides an attractive alternative over drug-based therapies but there are two major concerns with the application of stem cells: there must be an unlimited source of stem cells and the damaged area should be easily accessible for transplantation at the same time.

For scientist the great challenge at the moment is to develop a reliable and reproducible method to reconstitute the myelin sheath in animals and apply this model to rebuild the complete myelin architecture in patients with MS.

Experimental autoimmune encephalomyelitis, the animal model of multiple sclerosis

EAE is an inflammatory autoimmune disease, inducible in mice and resembles MS. Rivers with his colleagues performed the first EAE experiment and discovered the similarity between the human disease MS and the vaccination-induced encephalomyelitis (Rivers and Schwentker, 1935). EAE can be induced by systemic immunization with different myelin antigens (PLP, MBP or MOG) in different mouse strains (Ben-Nun and Cohen, 1982; Ben-Nun et al., 1981; Pettinelli and McFarlin, 1981). It can be induced by adoptively transferring a myelin reactive encephalitogenic $CD4^+$ T cell population but propagation of the disease by $CD8^+$ T cells has been observed two times so far (Huseby et al., 2001; Sun et al., 2001). According to a simplified view, after immunization, T cells, which bear myelin specific T cell receptors, get activated and clonally expand. Peripheral T cells require two different types of signals; the first one is antigen-

dependent signal; the second one is antigen-independent signal and it is provided by the co-stimulatory molecules of dendritic cells (DCs) (Becher et al., 2006). The most important co-stimulatory signal is the B7-CD28 receptor-ligand system. CD28 is expressed on the surface of T cells while its ligands belong to the B7 (CD80, CD86) family and they can be found on the surface of APCs. Co-stimulatory signaling is vital for T cell activation and in the absence of CD28-B7 interaction T cells might become anergic or unable to proliferate or can undergo apoptosis even in the presence of antigen stimulation. This process is called T cell priming/activation, which ultimately leads to the second, effector phase. In the later process, activated T cells traffic to the brain and spinal cord where they need to re-encounter with their cognate antigen on the surface of APCs in the context of MHCII molecules (Greter et al., 2005b). It has been shown that CNS resident APCs such as microglia do not function as local APCs and are not required for T cell entry and disease development. Oligodendrocytes cannot fulfill this function hence they cannot present antigen on the cell surface. It has been shown that a small population of $CD11c^+$ cells which is present at the blood brain barrier and meninges could be the responsible cell type, which presents the T cell cognate antigen in the context of MHCII (Greter et al., 2005b). This hypothesis is supported by the fact that in humans analogous $CD209^+$ cells can be found in healthy brain tissue which might suggest that antigen presentation could happen at the blood brain barrier, thus facilitating the entry of auto-aggressive T cells to the CNS (Hickey and Kimura, 1988). After CNS invasion T cells leave the blood brain barrier "open" and later a large number of circulating lympocytes readily gain access to the CNS. After having recognized their target antigen, in a short period of time they induce demyelination, which ultimately leads to ataxia and paralysis in immunized rodents.

T helper cell polarization

In 1986, Mosmann, et al. described that T helper lymphocytes can be subdivided into two major categories, T_H1 and T_H2, based on distinctive cytokine secretion patterns (Mosmann et al., 1986). $CD4^+$ T cells, producing IFN-γ, IL-2, lymphotoxin (LT) and TNFα have been defined as T_H1 and are responsible for cell-mediated immunity, defense against intracellular pathogens and for promoting the differentiation of $CD8^+$ T cells into cytotoxic killer cells (Hsieh et al., 1993; O'Garra and Robinson, 2004). IFN-γ activates macrophages and enhances their phagocytic activity in order to destroy microbes more efficiently.

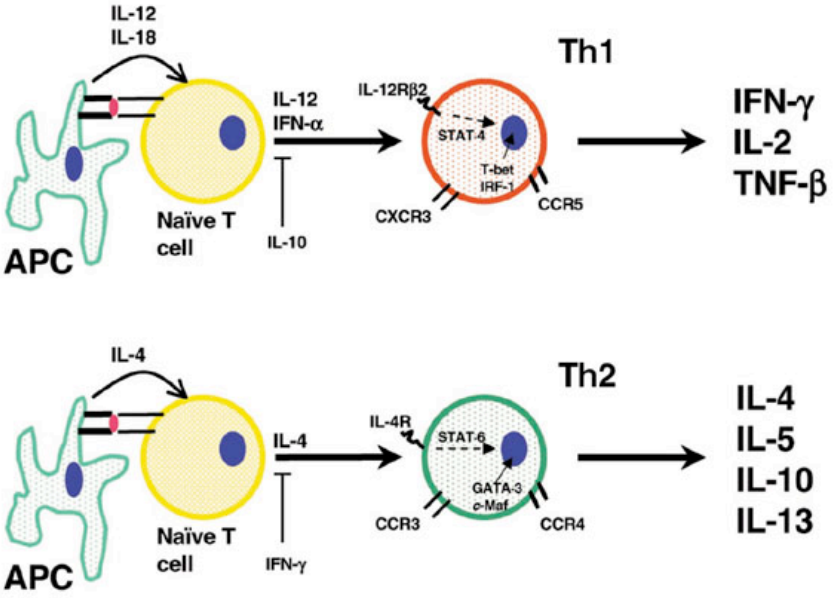

Figure 2. Schematic presentation of the development and effector function of T_H1 and T_H2 subsets

Cytokines secreted by antigen presenting cells initiate the differentiation of naive T cells towards different effector T helper subsets. T_H1 cells secrete IFN-γ as their hallmark cytokine and they require for their development the presence of IL-12 and IL-18 while the development of T_H2 cells is trigerred by IL-4. T_H1 cells play an important role in the regulation of antigen presentation and cellular immunity, while T_H2 cells are crucial mediators of allergies.
(Theofilopoulos et al. Arthritis Res 2001)

At the same time raise ability of APCs for antigen presentation by up-regulating MHC class II molecules. T_H1 cells preferentially express on the surface the receptor of IL-12 and the chemokine receptors CCR5 and CXCR3. Recently it has been shown that exclusively T_H1 cells express Tim-3 and it regulates the macrophage activation (Anderson et al.; Monney et al., 2002). Another newly described T_H1 specific cell surface complex is the CD94/NKG2, which might enhance the proliferation and IFN-γ production of T_H1 cells (Meyers et al., 2002).

T cells producing IL-4, IL-5, IL-10 and IL-13 are named T_H2 cells and play an important role in humoral immunity and the defense against extracellular pathogens (Coffman, 2006; Li-Weber and Krammer, 2003). Only T_H2 cells are able to activate resting B cells by secreting their hallmark cytokines, IL-4 and IL-13 (IFN-γ exerts an opposing effects on B cells). It has been shown that these two cytokines also trigger the accumulation of IgE antibody secreting B cells while the IL-5 secretion of T_H2 cells recruits mast cells and eosinophils, which are the typical cell types in

allergic type of inflammation (Cohn et al., 2004). T_H2 cells preferentially express CCR3, CCR4 and CCR7 on the cell surface.

T cell differentiation is regulated at many levels (MHCII-TCR interaction, cytokine signaling, co-stimulatory molecules, and transcription factors). T_H1 or T_H2 responses are induced depending on the pattern of cytokines that are present during the clonal expansion of antigen-specific T cells (during the priming phase). Naïve $CD4^+$ cells differentiate into polarized T_H1 and T_H2 during activation in the appropriate environment; for example, in the presence of IL-12 or IL-4 respectively.

IL-12, the T_H1 polarizing cytokine

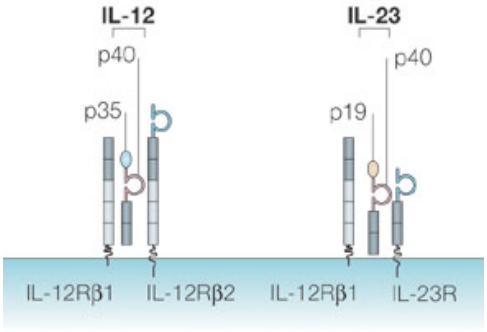

Figure 3. Structure and receptors of IL-12 and IL-23
IL-12 and IL-23 belong to the same IL-6 family of cytokines. Members of this family very often share receptor and/or cytokine subunits. p40 not just dimerizes with p35 to form IL-12 but together with p19 it gives rise to IL-23. Furthermore, IL-12 and IL-23 share receptor subunits as well. Both are produced by antigen presenting cells and act on cells such as macrophages, DCs and T cells thereby synchronizing the action of the two effector arms of the immune system. Both cytokines are thought to be involved in the orchestration o autoimmunity. IL-12 is held to be responsible for the generation of T_H1 cells while IL-23 is absolutely needed for the generation of pathogenic T cells.
Hunter et al., Nature Reviews Immunology 2005

IL-12 was first described in 1989 as a product of Epstein-Barr virus transformed human B cell lines, which induce IFN-γ production, activate NK cells (its first name was NK cell activating factor) and induce T cell proliferation (Kobayashi et al., 1989). IL-12 is a covalently linked heterodimer that is formed by a light chain of approximately 35 kDa (p35) and a heavy chain of approximately 40 kDa (p40) (Gately et al., 1991; Gubler et al., 1991; Wolf et al., 1991). The p35 subunit shows significant homology to other single-chain cytokines (IL-6, G-CSF) whiles the p40 heavy chain has similarity to the extra cellular domain members of the haematopoietic cytokine-receptor family (IL-6Rα chain). p35 is expressed constitutively while interestingly the production

of the p40 subunit is under tight regulation. The active form, p70 is secreted only if the same cell type simultaneously produces the two subunits although a biologically active p40 homodimer was characterized as well (Gillessen et al., 1995; Ling et al., 1995). Active IL-12 is secreted mainly by DCs and macrophages although it has been shown that neutrophils and monocytes can produce it under certain circumstances (Kobayashi et al., 1989; Ma and Trinchieri, 2001). Its production is primarily induced after stimulation of Toll-like receptors through their respective ligands such as lipoplysaccharide (LPS), peptidoglycan and bacterial (CpG) DNA (Medzhitov, 2001). Furthermore, in the primary immune synapse between T cell and APCs, CD40 engagement with its counterpart CD154 (CD40L) also stimulates IL-12 production (Cella et al., 1996).

The IL-12 receptor is a heterodimer and it is composed of IL-12Rβ1 and IL-12Rβ2 subunits. In order to bind IL-12 with high affinity both subunits should be co-expressed. IL-12Rβ1 subunit seems to be present more broadly while the β2 subunit is tightly regulated and specific in the case of $CD4^+$ T cells only for T_H1 cells (Rogge et al., 1999). The functional receptor of IL-12 can be found on several cell types such as activated T cells, NK-cells, macrophages and DCs (Gran et al., 2004b; Puccetti et al., 2002). Interestingly resting T cells do not express the receptor, it only appears on the surface after T cell receptor engagement (Desai et al., 1992). It has been shown that the expression of IL12Rβ2 is the regulated by IL-10 and TGF-beta and this regulation is part of the complex regulation network, which directs naïve T cells towards different fates. It seems that IL-10 mediated suppression of the receptor is the fine balance between effective protection against microbes and harmful systemic inflammation (Trinchieri, 2003).

IL-12Rβ2 is the signaling subunit and after ligand binding, the tyrosin residues on the intracellular part of the receptor get phosphorilated. This phosporilation subsequently leads to the activation of Janus kinase 2 (JAK2) and tyrosin kinase 2 (TYK2) which at the end activate different transcription factors such as STAT1, STAT3, STAT4 and STAT5 (Bacon et al., 1995a; Bacon et al., 1995b; Cho et al., 1996). Although it seems that only STAT4 mediates the biological effects of IL-12. This hypothesis is supported by the fact that $Stat4^{-/-}$ animals have decreased IFN-γ secretion, reduced NK-cell activity and impaired T_H1 response. Interestingly, these $STAT4^{-/-}$ T cells lack the expression of typical T_H1 receptors such as CCR5, IL-18R, E-selectin and P-selectin ligand. Recently another pathway was described which involves the activation of mitogen activated protein kinase (MAPK) p38 pathway. It seems that p38 directly interacts with STAT4 and activates it by phosphorilating one of its serine residues. Mutation of this residue prevents IFN-γ production and T_H1 differentiation. Taken together this, it suggests that both pathways are required for the full activation of STAT4 and to transmit proper IL-12 message. The targets of IL-12 are primarily T cells and NK-cells which in response to this cytokine release IFN-γ (Hsieh et al., 1993; Manetti et al., 1993).

T_H1 cells together with IL-12 were shown to mediate organ-specific autoimmunity and T_H2 cells were implicated in the pathogenesis of asthma and allergy (Bouma and Strober, 2003; Leonard et al., 1995; Lowes et al., 2007). In EAE, production of T_H1 cytokines by T cells has been shown to correlate with encephalitogenicity (Diab et al., 1997; Issazadeh et al., 1996; Issazadeh et al., 1995; Segal et al., 1998). Also, T cells recovered from the cerebrospinal fluid (CSF) of MS patients showed predominantly a T_H1 cytokine profile (Comabella et al., 1998; Drulovic et al., 1997; Nicoletti et al., 1996; van Boxel-Dezaire et al., 1999). These observations suggested the notion that EAE as well as MS are T_H1-mediated diseases. However, deletion of major T_H1 genes, such as IFN-γ and TNF-α, has surprisingly led to even more severe disease development, thus questioning the $T_H1/2$ paradigm in MS and EAE (Chu et al., 2000b; Frei et al., 1997; Willenborg et al., 1996). In the same line of thought it was believed that the T_H1 cytokine IL-12 plays a central role in autoimmune inflammation, specifically in the brain (Ozenci et al., 2001). But this was a misinterpretation, because one of the chains of the heterodimeric IL-12 is also part of a newly described, biologically active cytokine, IL-23 and it has recently become clear that IL-23 and not IL-12 is the critical factor in autoimmune inflammation of the CNS.

IL-23: The new player

The recently described biologically active cytokine IL-23 is a member of the IL-6 cytokine-family with discrete functions from IL-12 (Lankford and Frucht, 2003). IL-23 shows the common features of the type I cytokine super family such as the common four-helix bundle and its receptor has the haemotopoietin-receptor domain. It is, like IL-12, a heterodimeric protein comprised of two disulfide-linked subunits. In 2000, Kastalein and his colleagues identified p19 based on homology search for IL-6 family members and overall it has a 40% sequence identity to the p35 subunit of IL-12. In contrast to IL-12, which is composed of p35 and p40, IL-23 consists of the very same p40 but a unique p19 subunit. The p19 component is produced primarily by APCs such as macrophages and DCs. Its production is directly induced by CD40-crosslinking and indirectly by IL-1 and IFN-γ (van Seventer et al., 2002; Wesa and Galy, 2002). T_H1 cells have also been shown to express p19 mRNA; however, only activated antigen-presenting cells such as monocytes, macrophages and DCs concomitantly express p40 to form the biologically active cytokine (Ma et al., 1996; Yoshida et al., 1994).

Given the structural similarities between IL-12 and IL-23, it is not surprising that their receptors share subunits too. The IL-23 receptor is composed of two subunits such as IL-12Rβ1 and IL-23R, a shared subunit with IL-12 and a unique subunit of IL-23, respectively (Parham et al., 2002). The expression of IL-23R has been demonstrated on activated/memory T cells, several NK cell lines and at low levels on monocytes, DCs, inflammatory macrophages and microglia

infiltrating the CNS during autoimmune inflammation (Belladonna et al., 2002; Parham et al., 2002; Sonobe et al., 2008). Similar to the IL-12 receptor, the IL-23 receptor complex lacks intrinsic enzymatic activity. Instead, IL-12Rβ1 binds the Janus kinase (Jak) family member Tyk2, whereas IL-23R associates with Jak2. IL-23 binding to the IL12Rβ1/IL-23R complex leads to activation of JAKs through ligand-induced autophosphorylation and transphosphorylation of receptor-associated JAKs (Bacon et al., 1995a; Bacon et al., 1995b). The JAKs in turn phosphorylate the IL-23R at tyrosines located in the intracellular domain of the receptor subunit, forming docking sites for the STATs and potentially other signaling molecules. After phosphorylation, STATs dimerize and translocate to the nucleus where they activate target genes. However, in contrast to IL-12, IL-23 preferentially activates STAT3, with its binding to STAT4 being quite weak compared to that of IL-12, a fact that might explain why the biological effects of these cytokines are similar but not identical (Parham et al., 2002). IL-12-induced DNA-binding complexes contain only STAT4, whereas IL-23 induces several complexes containing STAT3, STAT1, and STAT4, which indicates that IL-12 and IL-23 may act on different target genes (Oppmann et al., 2000; Trinchieri, 2003; Wiekowski et al., 2001).

The most important producers of IL-23 are macrophages and DCs and its production can be induced by the presence of certain pathogens (E.coli, Bacteriodes vulgatus, Veillonella parvula, B. burgdorferi) and Toll-like receptor agonists, such as LPS and PolyI:C (Knauer et al., 2007). Peptidoglycan, a cell wall component of Gram-negative bacteria, can induce a more robust production of IL-23 by DCs than LPS (Morelli et al., 2001). Due to the presence of IL-23 receptor on APCs, IL-23 can bind to its producing cells and modulates their costimulatory function in an autocrine manner.

Role of IL-23 in the innate immune system

DCs and macrophages are the central sentinels of the immune system and they are able to govern the balance between ignorance, tolerance and immunity by interpreting the signals coming from the outside. It has been proposed that CD40L–CD40 interaction which is strong positive feedback for antigen presenting cell activation, might enhance the secretion of IL-23. Powrie and her colleagues studied the contributions of IL-23 to innate responses in the pathogenesis of inflammatory bowel disease (IBD). The development of IBD is highly dependent on the CD40-CD154 pathway. They used a T cell free environment in which an agonistic anti-CD40 antibody replaced the need for CD154-expressing activated T cells. After administration of anti-CD40 in a T cell-independent model (RAG animals) they observed an intestinal inflammation followed by systemic inflammatory response including elevated serum levels of pro-inflammatory cytokines, splenomegaly and wasting disease. They demonstrated that the systemic inflammation

was dependent exclusively on IL-12, while the local intestinal inflammation could be blocked by either p19 antagonizing antibodies or crossing RAG deficient mice with $p19^{-/-}$. In the intestine, a robust accumulation of DCs could be observed and they produced high amounts of IL-23. In response to local IL-23, T_H17 hallmark cytokines (IL-17A, IL-17F and IL-22) were secreted (Uhlig et al., 2006). The exact cellular source of these cytokines is still remains partially unidentified but most likely neutrophils, gamma-delta T cells and macrophages secrete them. After a series of transfer experiments, there is clear evidence that IL-23 does not exert its pathological effects by inducing the production of pro-inflammatory cytokines but it suppresses Treg differentiation in the intestine (Izcue et al., 2008; Laffont and Powrie, 2009; Siddiqui and Powrie, 2008).

Furthermore, a recent study revealed that during C. rodentium infection in a T cell independent model, IL-22 is induced by IL-23 and IL-22 secretion is critical for host survival during early phase infection. Most likely DCs are the major source of IL-22 in the intestine although the contribution of NK cells cannot be completely excluded (Coombes et al., 2007; Jaensson et al., 2008; Kullberg et al., 2006; Munoz et al., 2009; Zheng et al., 2008).

IL-23 in the regulation of T cell activity and auto-immunity

As a consequence of the structural and functional similarities between IL-12 and IL-23, it seemed likely that IL-23, similar to IL-12, would also have a function in the regulation of T_H1-cell response. However, it became apparent that these two cytokines have discrete roles in the regulation of T cells during autoimmunity and individual functions of these immunomodulators may be due to differential regulation of their expression levels and the use of different STAT homo- and heterodimers.

IL-12 has an established role in the induction of IFN-γ production and in the polarization of T cells to the T_H1 pattern meanwhile IL-23 plays a pivotal role in the induction of memory $CD4^+$ T cell proliferation and also sustains cell-mediated immune response by promoting the survival and effector cytokine production by $CD4^+$ memory T cells. In addition, IL-23 primes peptide-loaded DCs for promoting delayed-type hypersensitivity responses. IL-23 has similar actions on both $CD8^-$ and $CD8^+$ DCs to promote antigen presentation whereas IL-12 preferentially acts on $CD8^-$ DCs (Belladonna et al., 2002).

Historically, T_H1 cells/IFN-γ-mediated immune response were thought to mediate autoimmune diseases because $p40^{-/-}$ mice showed non-encephalitogenic phenotype indicating the pivotal role of IL-12 in the development of autoimmune mediated inflammation of the CNS. However, several studies questioned the casual link between autoimmune diseases and T_H1 or IL-12-IFN-γ cytokine axis. Deleting IFN-γ or its proximal components of its pathway (IFN-γR or STAT4) does

not impair EAE development in mice (Chu et al., 2000b; Ferber et al., 1996; Willenborg et al., 1996). Coinciding with the discovery of IL-23, discordant immune responses have been observed between $p35^{-/-}$ and $p40^{-/-}$ mice (Mattner et al., 1996). $p35^{-/-}$ mice are highly susceptible to EAE while $p40^{-/-}$ mice are completely resistant to the induction of EAE (Becher et al., 2002a; Zhang et al., 2003a). As far as T_H1 and T_H2 markers are concerned, T_H1 polarization is completely ablated in both $p40^{-/-}$ as well as $p35^{-/-}$ mice. This caused the dilemma that the dogma of T_H1 immunity as a prerequisite of encephalitogenicity was finally broken and no reliable pathogenicity marker has been put forward yet. Through studies using IL-23-deficient $p19^{-/-}$ mice and careful re-analysis of $p40^{-/-}$ mice versus $p35^{-/-}$ mice which develop severe EAE when immunized with MOG peptide, IL-23 has emerged as the key player in inflammatory autoimmune responses.

Mouse genotype	Phenotype	T cell polarization
Wt	Encephalitogenic	T_H1
$p35^{-/-}$ (IL-12)	Encephalitogenic (EAE more severe than in the wt)	T_H17
$p40^{-/-}$ (IL-12 and IL-23)	Non- encephalitogenic	T_H0
IL-12Rβ2 $^{-/-}$ (IL-12)	Encephalitogenic (EAE more severe than in the wt)	T_H17
IL-12Rβ1$^{-/-}$ (IL-12 and IL-23)	Non- encephalitogenic	T_H0
$p19^{-/-}$ (IL-23)	Non- encephalitogenic	T_H1
IFN-$\gamma^{-/-}$	Encephalitogenic (EAE more severe than in wt)	T_H17
$IL\text{-}23R^{-/-}$	Non- encephalitogenic	T_H0

Table 1. Observed EAE phenotype in different cytokine or cytokine receptor deficient animals

Since the discovery of IL-23 it was clear that it shows similar effects to IL-12 but it has its unique effector mechanism. Oppmann and his collegues showed that IL-23 induces the production of IFN-γ but lesser extent than IL-12 and interestingly it does not act on naïve T cells (Oppmann et al., 2000). This suggested the notion that the receptor is not expressed on inactivated, naïve T cells. Aggarwal and his colleagues showed that the receptor for IL-23 it only gets expressed on memory (CD44 high, CD62L low) T cells. They showed in their study that purified $CD4^+$ memory T cells after IL-23 exposure start producing significant amount of IL-17A. Their study was one of the most important milestones in the understanding of the biological role of IL-23. This paper paved the road towards the discovery of a completely new T helper subset, which later on dominated the field of auto-inflammatory disease research (Aggarwal et al., 2003b).

The discovery of IL-23 has led to the re-evaluation of the relationship between IL-12 and its role in the initiation of inflammatory diseases. The generation of all gene targeted mice made it possible to carefully re-evaluate the link between the autoimmune inflammation of the CNS and IL-12 and IL-23. It was already confusing from the very beginning that the $p40^{-/-}$ (IL-12 and IL-23 are missing) mice showed a contradictory phenotype to the $p35^{-/-}$ animals (Holscher et al., 2001). It became obvious that the p40 subunit is vital for the development of CNS inflammation while the deletion of the other IL-12 subunit, p35, surprisingly results in more exacerbated disease development (Becher et al., 2002a; Gran et al., 2002; Zhang et al., 2003a). Cua and his coworkers provided a resolution of this paradox by the generation of $p19^{-/-}$ animals, which made it possible to separate the actions of IL-12 and IL-23 *in vivo (Cua et al., 2003b)*. Studies of EAE demonstrated that IL-23 deficient mice were resistant to CNS autoimmune inflammation and they developed normal T_H1 responses. Studies with all cytokine mutant animals showed that the *in vivo* functions previously attributed to IL-12 for EAE are mostly induced by IL-23. Consistent with the finding of Aggarval, it became obvious that mice lacking IL-23, they are severely impaired in their capacity to develop IL-17-producing T lymphocytes and this finding provided the first evidence of a unique role for IL-23 in the regulation of a T cell effector function. This unique subset of $CD4^+$ T cells are characterized by production of IL-17 and crucially involved in the pathogenesis of certain autoimmune diseases and for their *in vivo* action they require the presence of IL-23 (Aggarwal et al., 2003b; Frucht, 2002).

It has been known that IL-12 induces factors, which are necessary for cytotoxicity such as IFN-γ, granzyme and FasL. In contrast, IL-23 stimulation leads to the expression of those genes, which are involved in long-term, chronic inflammation such as IL-17 and F, TNF-α, IL-6 and other pro-inflammatory cytokines. All these data suggested the fact that T cells differentiated in the presence of IL-23 could be a different and separate T cell subset, next to the previously described T_H1 and T_H2 cells and later was named as T_H17 cells (Langrish et al., 2005a).

IL-23 in other autoimmune diseases

The involvement of IL-23 and its receptor has been investigated extensively in the development of psoriasis. It is known that local administration or over-expression of IL-23 leads to erythema, acanthosis, hyperplasia and hyperparakeratosis, the typical hallmarks of psoriasis (Chan et al., 2006; Piskin et al., 2006; Zheng et al., 2007). It has been demonstrated that the level of IL-23 (measured at the mRNA level) in inflamed skin is much higher than in uninvolved skin. At the site of inflammation, most likely dendritic cells and keratinocytes produce it but its effects are mostly unknown. Several observations support the pathogenic role of IL-23:

- in concert with IL-1β enhances the production of antimicrobial peptides such as beta-defensin-2 (protein produced by keratinocytes in psoriasis) (Kanda and Watanabe, 2008)

- blocking TNF-α reduces IL-23 level and inhibits inflammatory infiltrates into the psoriatic skin (Zaba et al., 2007; Zaba et al., 2009)

- interfering with its production by different blocking antibodies, UV radiation or cyclosporine A ameliorates skin inflammation (Gottlieb, 2005; Haider et al., 2008; Piskin et al., 2004; Zaba et al., 2009)

The effector mechanism of IL-23 in psoriasis involves the action of Th17 cells, as in other auto-inflammatory processes. It is highly supported that Th17 cells are the key contributors to chronic skin inflammation by producing their hallmark cytokines especially IL-22 and IL-17F. These factors induce the activation of keratinocytes and the release of pro-inflammatory cytokines and chemokines by activated skin resident cells. This leads to the subsequent recruitment of other inflammatory cells to the site of inflammation thereby amplifying the immune response and resulting in clinical symptoms of psoriasis.

IL-17 cytokine family

The first member of the family, IL-17A, was discovered more than 15 years ago by Rouvier and his coworkers (Rouvier et al., 1993). Originally it was named as CTLA8 and was only subsequently renamed to IL-17A (Kennedy et al., 1996). At the moment six different cytokines belong to this family, and some of them have opposing effects to IL-17A. Homologues of this family can be found in various species, even in C. elegans. IL-17A is the prototypic member of the family; it is a disulfide linked homodimeric glycoprotein, contains the typical five conserved cystein residues which form the characteristic cystein-knob, exerts its action as a homodimer and shares no sequence homology with other known mammalian proteins. The other members of the family were discovered subsequently during the years based on homology search and degenerative PCR. The homology is the most apparent between IL-17A and IL-17F around 55% and others follow as: IL17B (29%), IL-17D (25%), IL-17C (23%) and IL-17E being the least related, sharing only 17% of homology at the primary amino acid sequence in humans (Korn et al., 2009; Weaver et al., 2007).

Only IL-17A and IL-17F are tightly linked and are localized on the same chromosome while the other family members are all located on different chromosomes. Initial reports described them as only homodimers but recent findings indicated that they might form heterodimers *in vivo*. So far no unique biological properties can be assigned to this IL-17A-F heterodimer (Chang and Dong, 2007).

Activated CD4+ T cells, the so-called T_H17 cells, mainly produce IL-17A and its close homologue IL-17F (Starnes et al., 2001). However, it has been reported that their expression does not appear to be limited to this T helper subset but $CD8^+$ T cells, NK cells, neutrophils, eosinophils and $\gamma\delta$ T cells express these cytokines. The fact that these two cytokines are produced by both the innate and adaptive immune system, suggests that they might orchestrate the action between innate and adaptive immune responses. Both have pro-inflammatory properties and they act on a broad range of cells. It has been found that they are potent inducers of cytokines (IL-1β, IL-6, IL-8, TNF-α) (Jovanovic et al., 1998), growth factors (GM-CSF, G-CSF), chemokines (CXCL1, CXCL6, and CXCL10) and metalloproteinases (Kanda et al., 2005; Numasaki et al., 2004). As they might connect innate and adaptive immunity, one of their main actions is to recruit neutrophils to the site of inflammation. Interestingly, it has been reported that IL-17A contributes to the formation of germinal centers together with follicular T cells, and enhances the production of IL-21 (Hsu et al., 2008; Tarlinton, 2008). The cellular sources of the other family members are less well characterized. It seems that IL-17B and IL-17C are not produced by lymphocytes and they are expressed mostly in pancreas, stomach, spleen, thymus and spinal cord (Yamaguchi et al., 2007). IL-17D is expressed by resting T cells and can be found in skeletal muscle and neuronal cells. The most distant relative, IL-17E which is called, IL-25 is the product of T_H2 cells and seems to play a role in allergic responses by inducing the expression of T_H2-type cytokines IL-4, IL-5 and IL-13 and chemokines such as CCL5 and CCL11 and recruiting eosinophils and basophils (Kempuraj et al., 2003). Moreover, it is inhibiting IL-17A secretion by up-regulating IL-13 by DCs and by limiting IL-23 production by APCs (Kleinschek et al., 2007; McHenga et al., 2008; Owyang et al., 2006; Wang et al., 2007).

Similarly to the IL-17A cytokines, their receptors constitute a unique family too. The first member of the family was described almost two decades ago and was named as IL-17RA. It is a single-pass trans-membrane protein with large intracellular tail and does not share homology with other known receptors except the other family members that are IL-17RB, IL-17RC, IL-17RD, and IL-17RE. Interestingly, all (except IL-17RA) of the receptors have splice variants, creating a huge variety of proteins. Some of them can be even secreted but retaining their ligand-binding properties thereby regulating the response to their ligands. The best characterized is the IL-17RA which is expressed by a huge variety of cell types such as hematopoetic cells, osteoblast, fibroblasts, endothelial and epithelial cells. It is the cognate receptor for IL-17A and IL-17F but it binds IL-17A with at least ten times higher affinity than IL-17F (Toy et al., 2006). In humans, it can form a heterodimer with IL-17RC, which is the cognate receptor for IL-17F. The *in vivo* function of this heterodimer is not yet known. After ligand binding, the receptor undergoes conformational changes, which mostly affects the cytoplasmic domain. The downstream signaling is not yet well characterized due to the lack of similarity to any other known receptor. So far it seems that IL-

17RA activates MAPK and the cascade subsequently involves NF-ΚB and TRAF-6 signaling (Gaffen, 2009).

IL-17RA together with IL-17RB forms the functional receptor for IL-25 (Gratchev et al., 2004), while the ligands of the other family members are still partially unknown. IL-17RB functions as the receptor for IL-17B and IL-17RE for IL-17C while the interacting partner of IL-17RD remains elusive.

The discovery of T_H17 cells and their role in autoimmunity

After the initial discovery of IL-23 and its impact on IL-17 production, it became quickly obvious that IL-17A is produced by a distinct subset of T helper cells, which is not overlapping with the already described ones. Under homeostasis, these cells reside mostly at barrier surfaces, most prominently at the gut epithelium, and their function is to protect the host from pathogens that attack through the epithelium (Marks and Craft, 2009; Steinman, 2007).

As previously discussed, T_H1 cells were held to be the responsible cell type for the development of autoimmune inflammation but the phenotype observed in IFN$\gamma^{-/-}$, IFN-γR $^{-/-}$, IL-12p40$^{-/-}$and IL-12p35$^{-/-}$ challenged the T_H1 driven organ-specific autoimmunity concept. Later on, it has been shown that none of the T_H1 driving cytokines (IL-12, IL-18) is vital for EAE development and all this contradicting data set led to the assumption that another subset of T cells might be required for the induction of organ–specific autoimmune diseases (Gutcher and Becher, 2007; Gutcher et al., 2006a). On the basis of their hallmark cytokine, these cells were subsequently called T_H17 cells. IL-23 emerged as a key player for the generation/maintenance of the T_H17 cell subset and it became obvious that it is indispensable for the development of tissue-specific auto-inflammation (Langrish et al., 2005a).

The importance of T_H17 cells in autoimmune inflammation has been proven in innumerous studies. First, Cua and his co-workers in an elegant study showed that IL-23 is essential for EAE development by the use of p19$^{-/-}$ animals. They demonstrated that the deletion of p19 subunit of IL-23 causes a drastic reduction in the number of IL-17-producing T cells in the CNS (Cua et al., 2003b). It was observed previously that IL12p35$^{-/-}$ animals instead of being EAE resistant, they rather developed more severe disease (Gran et al., 2002). At that time, nobody could provide an appropriate explanation to the observed phenomena but with the discovery of the T_H17 cell lineage, it became apparent that there is a reciprocal developmental pattern between IFN-γ^+ and IL-17$^+$ cells resulting in a more severe EAE development in IL-12-deficint animals. Thus, a positive correlation was established between the availability of IL-23 and IL-17-producing effector T cells and disease development, and a negative association was found between IL-12 and IFN-

γ^+ Th1 cells and EAE progression. In the inflamed CNS of IL-12p35$^{-/-}$ animals, the presence of T_H17 cells was overwhelming and this observation only intensified the notion about the pro-inflammatory role of this T cell subset. The connection between T_H17 cells and disease severity became more apparent after re-analyzing the previously observed EAE phenotypes of IFN-$\gamma^{-/-}$, IFN-$\gamma R^{-/-}$ animals. Iwakura and his collegues generated an IL-17A$^{-/-}$ mouse in order to investigate the impact of IL-17 in the development of EAE. They demonstrated in their study that IL-17 is important for the development of EAE but not vital (Komiyama et al., 2006). They observed that the lack of IL-17 delays the onset of EAE significantly however IL-17$^{-/-}$ mice exhibited the same incidence as wild-type although with milder disease scores. In accord with these findings, IL-17A$^{-/-}$ animals or neutralization of IL-17A *in vivo* showed a decreased disease severity in type II collagen induced arthritis and over-expression of IL-17 in the joints exacerbated disease (Lubberts et al., 2004; Nakae et al., 2003a; Nakae et al., 2003b; Stamp et al., 2004).

Data presented in several more studies further linked IL-17-expressing cells and autoimmune inflammation. In passive transfer experiments, PLP (CNS-antigen) specific T cells cultured in IL-23 enriched media caused disease in recipient mice whilst cells with the same specifity failed to induce EAE when they were cultured in the presence of IL-12 (Chen et al., 2006).

All these observations confirmed the encephalitogenic role for T_H17 cells in organ-specific auto-inflammation and strongly suggested that T_H1 and T_H17 represent distinct effector subsets, and the traditional view of the division of T helper subsets has to be revised.

Polarizing factors of the T_H17 cells

After this initial discovery that IL-17 producing cells might be the culprits of the development of tissue-specific autoimmunity, the mechanism by which T_H17 cells differentiate from naïve CD4$^+$ T cells drawn an increasing attention. It became obvious soon that IL-23 cannot induce the differentiation of naïve CD4$^+$ T cells into T_H17 cells *in vitro*. In 2005, it has been shown that the differentiation program of IL-17 producing cells diverges early from polarization program of T_H1 and T_H2 effector cells. It was an intriguing observation that T_H1 and T_H2 hallmark cytokines block T_H17 differentiation and the blockage of the T_H1 effector cytokine IFN-γ and T_H2 IL-4 enhances the differentiation of T_H17 cells. Also, the deletion of the signaling cascade components of IFN-γ and IL-4 enhances T_H17 polarization. It is notably to mention that in contrast to IL-4 and IFN-γ, which participate in a positive feedback loop in order to enhance T_H1 and T_H2 polarization, IL-17A and IL-17F fail to exert a similar effect (Harrington et al., 2005a; Park et al., 2005).

It quickly became apparent that TGF-beta and IL-6, two cytokines with opposing effects, are needed for the differentiation of IL-17-producing cells *in vitro (Bettelli et al., 2006; Weaver et al.,*

2006; Zhou et al., 2007). TGF-beta has been shown to contribute to T_H17 development by inhibiting the action of STAT4 and GATA-3 thereby blocking the differentiation pathways of T_H1 and T_H2 cells, respectively (Harris et al., 2007; Mangan et al., 2006; Mathur et al., 2007).

It was observed previously that IL-23 is not involved in the early differentiation of this pathogenic cell population and its receptor is present at low levels on the cell surface of naïve T cells. An interesting observation is that T cell receptor engagement in the presence of IL-6 up-regulates the expression of IL-23 receptor thereby facilitating the action of IL-23 in T_H17 polarization while TGF-beta induces the expression of a vital transcription factor. IL-6 animals are EAE resistance, which shows the dependence of T_H17 lineage differentiation on IL-6 (Eugster et al., 1998; Okuda et al., 1998; Samoilova et al., 1998). Interestingly, Korn and his colleagues showed that by blocking the generation of regulatory T cells in an IL-6 lacking environment, restores EAE susceptibility and suggests the fact that *in vivo* there is an alternative pathway for T_H17 generation (Korn et al., 2007). It has been speculated that after initial differentiation, terminally polarized T_H17 cells produce a huge amounts of IL-21, which can further amplify the differentiation process of T_H17 cells. Nurieva et al showed IL-21 deficiency leads to impaired T_H17 generation and results in protection against EAE (Nurieva et al., 2007). However the role of IL-21 in EAE is questionable. Later on, two other groups made conflicting observations showing that the absence of IL-21 or its receptor does not interfere with T_H17 polarization *in vivo* and the EAE phenotype of wild type and mutant animals is indistinguishable. Even more, they could observe that the lack of IL-21 leads to exacerbated disease development and seemed likely that IL-21 under physiological conditions limits EAE development (Coquet et al., 2008; Sonderegger et al., 2008b). IL-21 is a pleitropic cytokine, such as TGF-beta, influencing many different components of the immune system and this broad range of activities might contribute to the existing conflicting results.

After the initial description of differentiating factors needed for T_H17 polarization, more and more factors have been shown to participate in the fine-tuning of T_H17 differentiation. Another cytokine, IL-1β has been shown to be critically required for the early programming of T_H17 cell lineage and T_H17-driven autoimmunity. IL-1β has a well-established role in autoimmunity, it was shown that it promotes autoimmune joint destruction (Lubberts et al., 2004) and furthermore IL-1RI$^{-/-}$ mice fail to develop EAE (Schiffenbauer et al., 2000).

Chung and Sutton provided evidence for the role of IL-1β in T_H17 differentiation. They demonstrated that the up-regulation of IL-1R is an IL-6-dependent process in T cells and together with IL-23 it synergized to regulate the expression of transcription factors, IRF4 and Rorγt, which are vital for the lineage commitment of T_H17 cells (Sutton et al., 2006; Sutton et al., 2009).

Other cytokines are involved in the regulation of the T_H17 driven immune response. But in contrast to the previously describe cytokines, the following ones rather than enhancing the

encephalitogenic properties of T_H17 cells they limit their action/differentiation thereby under physiological conditions they fulfill protective rolls in autoimmunity.

One of these cytokines is IL-25 or IL-17E. This protein belongs to the IL-17 cytokine family but interestingly it has an opposing effect on T_H17 polarization than the other known members of this family. Originally it was described as T_H2 cytokine and was thought to have significant role in amplification/initiation of T_H2 responses (Fort et al., 2001). Cua and his colleagues found that IL-25 deficient mice show drastically increased EAE susceptibility and in line with this observation there is an elevated level of IL-23 at the periphery of these animals (Kleinschek et al., 2007). In the inflamed CNS, a higher proportion of T_H17 can be observed and treatment of mice with rIL-25 induced IL-13, which is a strong inhibitor of IL-23 and IL-1β thereby limits the differentiation of T_H17 cells (Wang et al., 2007).

Another IL-6 family member, IL-27 emerged recently as a negative regulator of T_H17 differentiation. IL-27 is typical family member of the IL-6 family; hence it is composed of the subunits, EBI3 (p40 related molecule) and p28 (p35 homologue). IL-27, similarly to its relatives, is produced by antigen presenting cells, mainly by DCs (Hunter, 2005). Its receptor is a heterodimer (gp130 and WSX1) and the responder cell population can be found in the innate (neutrophils, mast cells, monocytes) and adaptive (T, B and NK cells) immune compartment too. IL-27 has an ambiguous role in inflammation; initial articles emphasized the involvement of IL-27 in T helper cell function and being a pro-inflammatory cytokine (Colgan and Rothman, 2006; El-behi et al., 2009; Hunter, 2005). IL-27 induces the expression of T-bet, a critical transcription factor for T_H1 polarization, which in turn results in IFN-γ production and IL-12Rβ2 up-regulation on the cell surface (Yang et al., 2009). However, after the generation of the IL-27R$^{-/-}$ animal, it became obvious that T_H1 differentiation is only transiently affected and it was noted that IL-27R$^{-/-}$ mice developed a severe, multi-organ inflammation as a result of exacerbated T cell activation (Diveu et al., 2009; Ivanov et al., 2007). In line of this observation, it is not surprising that IL-27R$^{-/-}$ mice showed elevated EAE clinical scores and furthermore blockade of IL-27 worsens CIA (Stumhofer et al., 2006). It has been found that the lack of IL-27 is associated with elevated T_H17 cell activity in the CNS during EAE (Fitzgerald et al., 2007a). The most likely explanation is that IL-27 inhibits the effect of the pro-inflammatory cytokine IL-6, thereby potentially inhibiting T_H17 generation (Batten et al., 2006; Colgan and Rothman, 2006; Yang et al., 2008a; Yoshimura et al., 2006).

The recent discovery of the involvement of IL-35 in the regulation of T_H17 development has added another layer of complexity to this field. IL-35 belongs to the same family as IL-12, IL-23 and IL-27 and it is a product of regulatory T cells (Collison and Vignali, 2008). Collison and his colleagues were the first ones, identifying this cytokine as requirement of suppressor activity of T_{regs} and define it as an anti-inflammatory member of the IL-6 family (Collison et al., 2007). Later on, two more groups found direct evidence that IL-35 negatively regulates the expression of IL-17

and Rorγt thereby limiting inflammation although the direct mechanism of action remains elusive (Bettini and Vignali, 2009; Niedbala et al., 2007).

T_H17 cells produce another known cytokine, which is characteristic to this population although its function still remains poorly characterized. IL-22 belongs to the IL-10 cytokine family and its expression was initially linked to IL-17A and as in the case of IL-17, IL-23 stimulation is enough to induce robust amounts of IL-22 in memory T cells (Colonna, 2009). Interestingly the receptor for IL-22 is not expressed on the cells of the immune system but it is mostly expressed by keratinocytes and fibroblats (Xie et al., 2000). Unexpectedly, despite the fact that mostly T_H17 cells produce it (recently a new cell population has been coupled to IL-22 production in the intestinal system) (Aujla and Kolls, 2009; Wolk and Sabat, 2006) seems to have no pro-inflammatory function in EAE although deletion of IL-22 results in milder disease development in arthritis (Koenders and van den Berg; Kreymborg et al., 2007). Because of its action on keratinocytes, most of the attention was directed towards its role in the skin. It has been shown that it facilitates keratinocyte expansion and differentiations, which are hallmarks of psoriasis and it also participates in the production of inflammatory mediators (S100s) that enable augmentation of skin inflammation (Ivanov et al., 2006; Nograles et al., 2009).

T_H17 cell specific transcription factors

After defining the main soluble factors required for lineage commitment by T_H17 cells, the attention turned towards other characteristical properties of the T_H17 lineage. Cua with his colleagues performed an Affymetrix gene-array analysis of T_H17 versus T_H1 cells. This study helped to identify the main transcription factor of T_H17 cells. Among several transcription factor candidates, Rorγt was one of the most abundantly expressed genes in the T_H17 cell population and as expected under T_H1 polarizing condition, T-bet was highly up-regulated which is the main T_H1 transcription factor (Ivanov et al., 2006).

Rorγt belongs to the family of retinoic acid-related orphan nuclear hormone receptors together with Rorα and Rorβ (Jetten, 2009). Initially, it was only described to be expressed in the thymus and induce the expression of certain anti-apoptotic proteins in order to promote the survival of double positive thymocytes. Its function in differentiated, circulating T cells was completely unknown until it has been associated with T_H17 cells and was identified as their main lineage specific transcription factor. Its forced expression in $CD4^+$ T cells induced the hallmark cytokines of T_H17 cells such as IL-17A, IL-17F, IL-22 and other chemokines CCL1, CCR6 and CCL20. Interestingly it enhanced the expression of certain TGF-beta family members reassuring the pivotal role of this family in the development of T_H17 cells. All these data suggested that Rorγt is

involved in the polarization of the T_H17 lineage. In line with this, T cells derived from Rorγt deficient mice showed a drastically reduced ability for IL-17 production under *in vitro* conditions that favored their polarization. Later on, retroviral transduction has proven that Rorγt delivery restores encephalitogeinc properties of T cells (Ivanov et al., 2006). *In vivo* experiments showed that it has a vital function in EAE induction because Rorγt deficient mice showed a significantly ameliorated disease development and in the CNS of afflicted animals the T_H17 population was highly reduced although not completely absent. Because of this incomplete inhibition of EAE it was hypothesized that other transcription factors might contribute to T_H17 differentiation (Martinez et al., 2008).

Yang and his coworkers reported that another family member, Rorα, is highly induced by TGF-beta and IL-6 in a STAT3 dependent manner in T_H17 cells. In the absence of this transcription factor, IL-17 production is reduced and a double deficiency together with Rorγt entirely abolished T_H17 polarization and resulted in a complete EAE resistance. Therefore, it seems likely that Rorα is another specific transcription factor that directs T_H17 lineage polarization (Sundrud and Rao, 2008; Yang et al., 2008a; Yang et al., 2008b).

As the time passed, more and more transcription factors became acquainted to IL-17–producing cell polarization. Schraml et al identified another transcription factor, namely Batf, and revealed that Batf deficient mice fail to develop autoimmune inflammation of the CNS although they are able to mount proper T_H1 and T_H2 responses. They showed a defect in T_H17 differentiation and $Batf^{-/-}$ cells failed to induce the expression of factors known to be required for T_H17 polarization such as IL-21 and Rorγt. Their results demonstrated that Batf plays a critical role in T_H17 differentiation (Martinez and Dong, 2009; Schraml et al., 2009).

Another group identified the transcription factor of the alternative differentiation pathway of T_H17 cells, which involves IL-21. They showed that IRF4 is absolutely needed in a T cell intrinsic manner for the production of and responsiveness to IL-21. The absence of IRF4 results in reduced production of IL-17 and T cells fail to up-regulate IL-23R thereby become unable to maintain their characteristical properties (Brustle et al., 2007; Chen et al., 2008; Huber et al., 2008).

Last but not least, another transcription factor has been linked to T_H17 development, namely the aryl hydrocarbon receptor (AhR). This receptor recognizes a big variety of small xenobiotic and natural molecules. AhR is best known for mediating dioxin toxicity, and recently it has been shown to be involved in mediating immune responses under normal physiology (Esser et al., 2009). T_H17 cells express high levels of AhR, although it is not required for T_H17 differentiation, most likely it promotes further functional differentiation of this lineage (Kimura et al., 2008). Its presence is pre-requisite for IL-22 expression but IL-17production seems not to be dependent on this transcription factor. It has been shown that activation of this receptor leads to earlier onset

and more severe disease development of EAE (Esser et al., 2009; Kimura et al., 2008; Quintana et al., 2008; Veldhoen et al., 2008). The connection between a receptor, which recognizes environmental pollutants, and T_H17 cell lineage, raises intriguing possibility regarding the potential of such a factor to augment autoimmune conditions such as EAE/MS and CIA/RA. As it is discussed earlier that environmental factors might contribute to the development of MS, further studies should provide a better understating of disease development.

T_H17 cells, are they really pathogenic?

As it is discussed previously there is huge body of evidence that shows the pivotal role of IL-17-producing cells in autoimmunity. However, recently a number of publications questioned the pathogenic properties of IL-17-producing cells, especially in EAE. Haak and his co-workers provided solid evidence that deletion of IL-17A does not have a major impact on EAE development, contradicting to the previous findings. Furthermore, they convincingly showed that either the lack of IL-17A (gene deletion) and IL-17F (inhibition by blocking antibody) together or over-expression of IL-17A by $CD4^+$ T cells have, at the best, marginal impact on EAE clinical score (Haak et al., 2009a; Hofstetter et al., 2005a). Other groups, in line with the previous observation, demonstrated that depletion of T_H17 cells prior to adoptive transfer does not prevent EAE development, once again, questioning the vital contribution of T_H17 cells to disease development (Koenders and van den Berg).

It is undeniable that T_H17 cells can be found at the site of inflammation but their presence does not necessarily mean that they possess encephalitogenic properties. As it has been shown previously, deletion of IL-17/IL-22 cytokines has only minor or no impact on EAE; blocking the IL-21 pathway does not interfere with T_H17 polarization and EAE development. From all this contradicting data we can state only one thing: more in-depth research is needed in order to demystify the exact contribution of T_H17 cells in autoimmunity and to reveal another yet un-described pathogenic molecule which has non-redundant functions in EAE development.

T_H17 cells in infectious disease

In recent years, the role of T_H17 cells was mostly emphasized in the context of autoimmunity but should not be forgotten their vital contribution to host protection against extracellular parasites. The receptor of IL-17 is widely expressed and after receptor engagement the expression of chemokines, colony stimulating factors and pro-inflammatory cytokines is induced. These factors in turn induce the recruitment of neutrophils and other myeloid cell to the site of infection, which is the hallmark of infectious diseases (Ferretti et al., 2003; Ye et al., 2001). It has been shown that APCs, especially DCs, in response to certain pathogens, Klebsiella pneumoine, Bordatella pertussis and M. tuberculosis, produce high amounts of IL-23 which subsequently maintains/activates the IL-17-producing T cell lineage (Andreasen et al., 2009; Chen et al., 2007). T_H17 cells can be activated by cell wall components of yeast and fungi such as zymosan, dectin-1 and beta-glucan (Curtis and Way, 2009; Happel et al., 2005).

These observations suggest that T_H17 cells play a central role in the protection against extra cellular bacteria and fungi, while T_H1 and T_H2 cells protect the host against intracellular pathogens and helminthes. T_H17 cells are rapidly recruited to the site of inflammation (faster than other effector T cell subsets) and by producing chemokines and other factors, they attract other T helper subsets to the site of inflammation, thereby serving as a bridge between innate and adaptive immunity (Ouyang et al., 2008).

Regulatory T cells

It has been proposed already in the late 70s that T cells could act as regulatory cells and they are able to adjust the strength of immune responses by producing soluble factors. However, the mechanism behind this suppression was not clarified thereby research within this field was abandoned. For long time, the existence of suppressor T cells was nothing else than a myth in immunology until when in the early 90s after a series of adoptive transfer experiments it became apparent their existence. These T cells have been identified by their expression of CD4, the IL-2receptor alpha chain (CD25) and their transcription factor FoxP3 (Chen et al., 2003; Wu et al., 2006). Later on, it became clear that FoxP3 expression is necessary and sufficient for the development of regulatory T cells. It has been shown that regulatory T cells can be generated in the thymus, they are called natural T_{regs}, while T_{regs} at the periphery are called adaptive T_{regs}. nT_{regs} are exclusively generated in the thymus, they bear receptors which recognize self-antigens, for their activation they require additional CD28 stimulation and for their generation TGF-beta, IL-2 and IL-15 are necessary (Komatsu et al., 2009; Malek and Bayer, 2004; Sakaguchi, 2005).

Differentiation of iT_{reg} cells takes place in the peripheral lymphoid system (GALT, spleen, lymph nodes) and in exceptional cases, in chronically inflamed tissues (Thornton, 2005). They recognize not just self-antigens but allergens, and commensal microbiota derived antigens. CTLA-4 co-stimulation is needed for their proper function, and IL-2 and TGF-beta are the vital factors for their maturation (Frisullo et al., 2006).

FoxP3 is vital for proper regulatory T cell function

As it is mentioned earlier, FoxP3 is essential for the development of regulatory T cells and in mice FoxP3 is a good phenotypic marker of T_{regs} together with CD25. The discovery of FoxP3 started with a spontaneous mutation, which led to the scurfy phenotype and later on the gene responsible for the scurfy disease was identified as FoxP3 (Brunkow et al., 2001; Godfrey et al., 1994). These mice exhibit a series of autoimmune features including ear thickening, leukocyte infiltration into the liver and skin, over-production of pro-inflammatory cytokines and early death (Shull et al., 1992). A series of experiments enforced the notion that FoxP3 is vital for the conversion of naïve T cells towards regulatory phenotype. This notion was strengthened by the failure of $FoxP3^{-/-}$ mice to develop functional suppressor T cells. These animals developed a scurfy-like phenotype, which could be rescued by adoptive transfer of T_{regs} from a wild type animal. Moreover, forced over-expression of FoxP3 in $CD25^{-}$ T cells initiated the differentiation of these cells towards the regulatory phenotype. It is unquestionable that in the absence of FoxP3, T cells are unable to keep inflammation under control although the precise mechanism by which it is achieved still remains elusive (Fontenot et al., 2003; Hori et al., 2003).

After the identification of regulatory phenotype, the next step was to reveal the mechanism by which they regulate the immune response. It has been proposed passive and active regulatory mechanisms. It is known that these regulatory T cells express the trimeric, high-affinity IL-2 receptor complex. It has been proposed that by expressing the high-affinity IL-2R, regulatory T cells soak up IL-2 from the local environment thereby slowing down the proliferation of effector T cells (Malek and Bayer, 2004). This is unlikely to be the major effector mechanism of regulatory cells, most likely active processes play the major role in suppression. Two anti-inflammatory cytokines have been implicated in the active suppressor mechanism by T_{regs}, namely TGF-beta and IL-10 (Fontenot and Rudensky, 2005; Ziegler, 2006).

TGF-beta and its connection to regulatory T cells

TGF-beta is a regulatory cytokine with essential role in immune responses and it is produced by a big variety of cell types of the body. Three homologous isoforms can be found in mammals with very similar *in vitro* properties. The secreted protein is not yet biologically active, it has to be cleaved and after cleavage it can bind to its receptor. Its receptor is a heterotetramer composed of two ALK5 subunits, and two TGF−betaRII parts. The receptor complex only initiates cell signaling after binding a TGF-beta dimer. The signaling pathway involves many intracellular proteins, among them the most prominent ones are the following: Smad2, 3, 4 and 7, MAPK and PI3K.

It is well known that TGF-beta exerts the biggest impact on T cells interfering with proliferation, differentiation and survival. Its importance in the immune system is underscored by the finding that TGF-beta-deficient mice show a serious, systemic inflammatory disease, which leads to their early death, approximately four weeks after birth (Marie et al., 2006; Shull et al., 1992). TGF-beta predominantly expressed by leukocytes, and later studies revealed that regulatory T cells express this protein as an effector cytokine. TGF-beta secreted by $CD4^+$ $CD25^+$ T cells is supposed to suppress T_H1 and T_H2 differentiation (Gorelik et al., 2002). Interestingly, TGF-beta mediated inhibition of differentiation even occurs in the presence of IL-2, but in this case T cell proliferation remains unaffected. TGF-beta is likely to block the expression of T-bet, the master transcription factor of T_H1 cells, thereby inhibiting the expression of IL-12Rβ2 subunit. TGF-beta reduces STAT4 expression, the signal transducer of IL-12 (Gorelik et al., 2002). It has been shown that by blocking different members of the IL-12 signaling pathway, TGF-beta can interfere at certain stages of T_H1 differentiation (Gorelik et al., 2000; Lee et al., 2009). Several reports showed that TGF-beta inhibits the development of T_H2 cells by blocking the T_H2 master transcription factor, GATA-3 (Gorelik et al., 2000). Most likely, this inhibition is achieved by the blockage of calcium influx although the precise complete mechanism of this inhibition remains still unclear. Its role in MS and in EAE is controversial but several studies indicate its protective role. Early publications showed that the administration of rTGF-beta *in vivo* or pre-treatment of antigen-specific encaphlitogenic T cells with TGF-beta *in vitro* can prevent the development of EAE (Johns et al., 1991; Kuruvilla et al., 1991; Racke et al., 1992; Racke et al., 1991). It has been observed that during remission TGF-beta mRNA level increased and neutralization by blocking antibodies enhanced clinical severity of disease (Johns and Sriram, 1993). Taken together all these data, it became obvious that it plays an essential role in protection in EAE although it should be not forgotten that together with IL-6 contributes to the generation of the supposedly pathogenic T cell population, the T_H17 cells.

IL-10 in immune regulation

As it is discussed previously, TGF-beta appears to have a dual role in immune regulation; on one hand, it participates in the generation of encephalitogenic T_H17 cells while in the other hand, it has anti-inflammatory properties. IL-10 produced by regulatory T cells plays an essential role in down-modulating inflammation and limiting the antigen driven immune response (Boxel-Dezaire et al., 1999). Originally it was described as a T_H2 cytokine but later on it became apparent that is one of the key factors produced by regulatory T cells in order to control tissue inflammation (Bettelli et al., 1998; Segal et al., 1998). Interestingly regulatory T cells are not the only ones producing this factor. Several groups observed that T_H17 cells produce this cytokine and IL-6 and TGF-beta are required for IL-10 production. It is likely that IL-10 functions as a break in order to prevent the escalation of inflammation (Lochner et al., 2008; Noguchi et al., 2007). Its modes of action most likely involve the blockage of B7/CD28 co-stimulatory pathway and it impairs DC maturation thereby leading to reduced MHCII presence. It is suggested that the level of IL-10 correlates with the strength of the inflammatory signal (McGeachy et al., 2007; Stumhofer et al., 2007).

The plasticity of T_H17 cells

T cell populations defined by the presence of CD4 or CD8 markers are inflexible because their fate is determined during ontogeny in the thymus. In contrast, it is getting increasingly clear that T cell populations defined by their function and by the expression of a particular transcription factor are not necessary stable. One of the best examples for T cell lineage plasticity is the developmental plasticity of T_H17 cells and regulatory T cells (O'Connor et al.; Zhou et al., 2009c; Zhu and Paul). Both cell populations require TGF-beta for their development, and this TGF-beta requirement provided the first evidence that these two cell lineages might be connected developmentally. T_H17 cells are in need of several transcription factors for their development, but it seems that Rorγt and to lesser extent Rorc plays the central role in their development. Regulatory T cells generated at the periphery require the action of FoxP3 for their development. Surprisingly, antigen-activated naïve T cells in the presence of TGF-beta are induced to co-express FoxP3 and Rorγt at the beginning of differentiation (Lee et al., 2009). As the differentiation proceeds, T_H17 cells subsequently down-regulate FoxP3 while in regulatory T cells the expression of Rorγt is being inhibited. Recently the interaction between the transcription factors has been revealed which provides a mechanistic basic for a better understanding of this antagonistic competition. It was demonstrated that Rorγt and Rorc are interacting partners of FoxP3, and their transcriptional activity is inhibited after FoxP3 binding (Zhou et al., 2008). Rorγt

binds to the motif encoded by exon 2 of the FoxP3 gene and thereby FoxP3 can directly repress T_H17 differentiation (Ichiyama et al., 2008). Another layer of complexity is added to the well-balanced system with a FoxP3 splice variant, which does not contain exon 2 but seems to be biologically active (Ziegler, 2006).

Interestingly, the concentration of TGF-beta influences the dominance of the transcription factors. At high concentrations of TGF-beta, the function of Rorγt is inhibited by an increased expression of FoxP3, while at low concentration, TGF-beta in concert with IL-6 overrides the blocking effect of FoxP3 thereby facilitating the completion of the T_H17 transcriptional program. The best example for this plasticity can be observed in the gut where DCs during homeostasis favor the development of regulatory T cells by producing retinoic acid (Afzali et al.; Correale et al., 2009; Mucida et al., 2009; Osorio et al., 2008). However, when DCs sense the danger of microbial intrusion, they get activated and start producing pro-inflammatory cytokines such as IL-6 instead of retinoic acid (Coombes et al., 2007). In this cytokine microenvironment naïve T cells diverted away from regulatory T cell pathway and they preferentially differentiate towards the T_H17 cell lineage (Sundrud and Rao, 2008).

Colony stimulating factors and their role in inflammatory processes

Colony stimulating factors such as M-CSF (macrophage colony-stimulating factor or CSF1), GM-CSF (granulocyte-monocyte colony stimulating factor or CSF2) and G-CSF (granulyocyte stimulating factor) were originally only described as haematopoietic-cell growth factors but recently the original observation has been revised and their role in inflammatory processes became more and more apparent. Initially, it was observed that colony-stimulating factors were able to induce the differentiation and maturation of granulocytes, macrophages and dendritic cells from undifferentiated bone-marrow stem cells *in vitro* and *in vivo*. Later on, it became clear that they not just have impact on undifferentiated cells but they can act on already mature myeloid cells. The most attention has been paid to GM-CSF that as its name implies is able to induce the differentiation of granulocyte and macrophage colonies out of precursor cells. It was thought that because of its ability, GM-CSF is the main myelopoietic growth regulator under steady state conditions. Surprisingly, although being the key factor in the differentiation of myeloid cells, its level in the circulation is low and deletion of GM-CSF does not affect negatively the number of myeloid cells in the circulation.

GM-CSF is a secreted, single chained glycoprotein. Its receptor is composed of two subunits; the alpha subunit is responsible for ligand binding whilst the beta subunit is the signaling partner of the receptor complex. It has been shown that after successful ligand binding, at least three different signaling pathways are initiated. Similarly to the IL-6 cytokine family, the JAK-STAT pathway is involved in the transmission of information to the nucleus. Next to the JAK-STAT pathway, the mitogen activated protein kinase and the phosphoinositide-3 kinase pathway are playing an important role in the signal transduction of GM-CSF.

GM-CSF is secreted in response to several inflammatory stimuli such as LPS, TNF-α, IFN-γ, IL-1 by a big variety of cells such as fibroblast, muscle cells, mast cells, macrophages, endothelial cells and most importantly activated $CD4^+$ T cells. The receptor can be found on the surface of almost on all previously mentioned cell types, with one exception, $CD4^+$ T cells.

It has been shown by gene deletion studies that in its absence the generation of alveolar macrohages is compromised which leads ultimately to pulmonary alveolar proteinosis (Carey and Trapnell). A recent study showed its involvement in the differentiation of invariant natural killer cells during thymic ontogeny. Its elevated level in the blood leads to the mobilization of monocytes and neutrophils from the bone marrow to the systemic circulation. *In vitro* studies showed that stem cells cultured in GM-CSF containing media develop either towards inflammatory DCs or classically activated macrophages. The adherent macrophage population in

a mature state produces pro-inflammatory cytokines such as IL-23, TNF-α, IL-6 and IL-12 after LPS stimulation.

GM-CSF in autoimmunity

GM-CSF can be found at the site of inflammation at higher amounts and it is thought to be part a network, which allows the communication between myeloid cells and neighboring cells during inflammation. Mature myeloid cells after being primed with GM-CSF for inflammatory stimuli, they start producing pro-inflammatory cytokines, which largely contribute to the exacerbation of a local inflammation (Hamilton, 2008b). In 2001, McQualter and his colleagues showed that GM-CSF is vital for EAE development and mice with a disrupted gene fail to develop clinical signs of MOG_{35-55} induced EAE (McQualter et al., 2001). Interestingly, accumulation of GM-$CSF^{-/-}$ T cells can be observed during autoimmune inflammation in the CNS but these T cells show a significantly reduced proliferative response to MOG_{35-55} restimulation and GM-CSF-deficient splenocytes secrete significantly lower amounts of IFN-γ and IL-6. Administration of rGM-CSF or its over expression by retroviral vectors in inflammatory T cells restores EAE susceptibility while the use of blocking antibodies significantly ameliorates disease progression (Campbell et al., 1997; Kroenke et al., 2008a; Marusic et al., 2002; Ponomarev et al., 2007). Another group showed that GM-CSF production solely by encephalitogenic T cells is sufficient to induce inflammation in the central nervous system and plays a key role in the activation of microglial cells. It has been suggested that GM-CSF produced by T cells is essential for the recruitment of peripheral macrophages and ultimately for the development of clinical signs of EAE (Ponomarev et al., 2007).

Kopf et al. by using a different type of autoimmune model, experimental autoimmune myocarditis, proposed a mechanism by which GM-CSF contribute to autoimmunity. They suggested that innate GM-CSF is essential for IL-6 and IL-23 production by dendritic cells. These two cytokines play a pivotal role in the differentiation/survival of pathogenic T_H17 cells *in vivo*. They showed that in the absence of GM-CSF, IL-6 production is drastically decreased which subsequently impairs the generation of IL-17-producing T cells although T_H1 cells remain unaffected. Moreover, they observed that GM-CSF promoted auto-inflammation by enhancing IL-6-dependent survival of antigen specific $CD4^+$ T cells (Sonderegger et al., 2008a).

Gene targeting

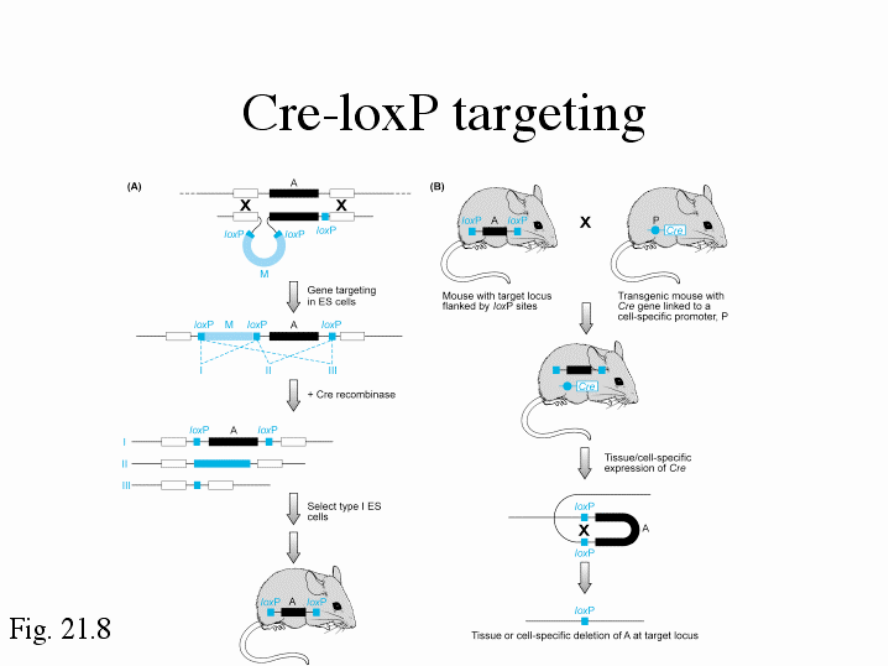

Figure 4. Schematic representation of the generation of a conditionally targeted mouse using the CRE-loxP system

The artificially modified gDNA containing the loxP-flanked exon/s is integrated into the mouse genome by homologous recombination. The animal carrying the mutant allele is crossed to a Cre expressing strain, which ultimately results in tissue specific removal of the desired gene.

Strachan and Read, Human Molecular Genetics 2, Wiley-Liss, New York, 1999

Simple in theory, difficult in practice. Understanding the *in vivo* function of a gene could be achieved by either inactivating or modifying it by mutation and subsequently studying the consequences of the mutation in the mutant organism. In mice, before gene targeting, mutation generation was a really time consuming and limited approach due to the rare obviously manifesting phenotypes (Kuhn and Schwenk, 1997). Gene targeting allows us to selectively remove, switch on/off or replace genes. In its original form, gene targeting meant the inactivation of a certain gene in the genome of embryonic stem cells (ES) by homologous recombination. ES cells are totipotent cells and in a lucky case they can be participating in the formation of the mouse germ-line, thereby allow the transmission of the mutation to the next generations. The classical way of gene targeting was robust method with all its advantages and disadvantages. From one hand it resulted in the inactivation of genes (which was desired) but on the other hand

because the mutation was present in all cells of the body from the very beginning of ontogeny, very often yielded complex phenotypes and early lethality (Mak et al., 2001).

In order to investigate the exact function of a gene, a method was desirable, which was able to regulate/restrict the mutation to certain cell types of the mouse and to certain time points during development. This desire has led to the introduction of conditional gene targeting by Rajewsky about 15 years ago. This strategy allows the researcher the introduction of targeted mutations in a cell type-specific and/or inducible fashion. This can be achieved by flanking one or several crucial exons of the gene with recognition sequences for a site-specific recombinase and later on this protein is expressed by a transgene in a cell type-specific or inducible fashion (Kolb, 2002; Kwan, 2002). This targeting results in a functionally intact allele of a gene but subsequently it can be deleted by the use of the Cre-loxP system (Le and Sauer, 2000). This system originates from the P1 bacteriophage and cannot be found in the native mouse genome, hence it has to be introduced artificially. The loxP sites are specific 34-base pair long sequences consisting of an 8-bp core sequence, where recombination takes place, and two flanking 13-bp inverted repeats. The orientation of the loxP sequences is crucial in order to determine whether deletion, translocation or inversion happens. Typically the loxP and Cre-containing mouse strains are developed separately and crossed together to achieve a Cre-loxP mouse strain. There is a big variety of Cre strains available, some of them being tissue-specific while others are inducible. Inducible Cre strains contain a modified Cre protein, which is non-functional till a certain compound (tamoxifen, doxyciclin) is administered at a desired time point during ontogeny resulting in the excision of the loxP flanked DNA segment (Mishina and Sakimura, 2007; Schmidt-Supprian and Rajewsky, 2007).

Results

GM-CSF secretion by TH cells is dependent on RORγt and essential for autoimmune neuro-inflammation

Laura Codarri, Gabor Gyülveszi and Burkhard Becher

(submitted)

IL-23-driven encephalo-tropism and TH17 polarization during CNS-inflammation

Gabor Gyülvészi, Stefan Haak and Burkhard Becher

European Journal of Immunology, 2009

Generation of mice with conditionally targeted il23r locus

(in progress)

GM-CSF secretion by TH cells is dependent on RORγt and essential for autoimmune neuro-inflammation

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Abstract

During autoimmune neuro-inflammation, helper T (T_H) cells initiate tissue damage and neurological impairment. Initially, IFN-γ-producing T_H1 cells were thought to be the driving force behind the inflammation seen in multiple sclerosis (MS) (Gutcher and Becher, 2007). It was later found that the deletion of IFNγ and the T_H1 inducing cytokine IL-12 led to exacerbated disease development in experimental autoimmune encephalomyelitis (EAE) (Becher et al., 2002b; Chu et al., 2000a; Cua et al., 2003a; Gutcher et al., 2006b). Now, it is widely held that IL-17-secreting T cells (T_H17) rather than T_H1 cells, are the main encephalitogenic population in autoimmune inflammation (Aggarwal et al., 2003a; Park et al., 2005), but to this day, none of the known T_H17 signature cytokines (IL-17A, IL-17F, IL-22, IL-21) are mandatory for the development of EAE (Haak et al., 2009a; Hofstetter et al., 2005b; Kreymborg et al., 2007; McGeachy et al., 2007; Sonderegger et al., 2008b). Nevertheless, IL-23 as well as the T_H17 transcription factor RORγt elicit an encephalitogenic program, which leads to the production of a so far unknown encephalitogenic factor or combination of factors.

Here we describe that GM-CSF fulfills all of the requirements of such an encephalitogenic cytokine. It is secreted by CNS-invading auto-aggressive T_H cells, its production is dependent on the activity of the IL-12/23 receptor complex and RORγt. Conversely, IFN-γ, IL-12 and IL-27 inhibit RORγt expression and the secretion of GM-CSF. We demonstrate that GM-CSF not only marks the population of highly pathogenic T_H cells, but that in contrast to IL-17 or IFN-γ, it exerts a non-redundant function in autoimmune T cell pathogenicity *in vivo*.

We and others analyzed the molecular signature of encephalitogenic T cells differentiated in presence of IL-23, and in addition to the above mentioned T_H17 cytokines, GM-CSF was found to be induced by IL-23 (Kreymborg et al., 2007; Kroenke et al., 2008b; Langrish et al., 2005b). In mixed bone-marrow (BM) chimeras, T cells unresponsive to IL-23 are impaired in their capacity to invade the CNS and do not acquire encephalitogenic properties (Gyulveszi et al., 2009; McGeachy et al., 2009b) highlighting that IL-23 endows T cells with the capacity to invade the CNS and to initiate tissue damage. Moreover, in vivo, IL-23 unresponsive T cells are unable to secrete IL-17 (Gyulveszi et al., 2009; McGeachy et al., 2009b), yet IL-17A and IL-17F have only a limited impact on the encephalitogenic potential of T_H cells (Haak et al., 2009a; McGeachy et al., 2007). GM-CSF deficiency or neutralization has been demonstrated to render mice completely resistant to the induction of EAE (King et al., 2009; McQualter et al., 2001; Ponomarev et al., 2007). T cells, the major source of GM-CSF during inflammation, were also implicated to deliver GM-CSF into the CNS, where it activated resident microglial cells and DCs (King et al., 2009; McQualter et al., 2001; Ponomarev et al., 2007). However, in the context of T cells polarization and IL-23-driven encephalitogenicity, the role of GM-CSF has not been studied.

In order to investigate whether GM-CSF is in fact the encephalitogenic factor secreted by IL-23 polarized T_H cell *in vivo*, we generated two different groups of mixed BM-chimeras. $RAG1^{-/-}$ mice in one group were reconstituted with a 1:1 mixture of IL-12R$\beta1^{-/-}$ and wt BM, while the other group was generated by reconstituting $RAG1^{-/-}$ with a mixture of IL-12R$\beta2^{-/-}$ and wt BM. This experimental setup enabled us to study the function of cytokine receptor deficient and wt cells in the same animals during inflammation. The IL-12R$\beta1^{-/-}$ T cells are unresponsive to IL-23 and IL-12, whereas IL-12R$\beta2^{-/-}$ T cells are unresponsive to IL-12 alone. After engraftment, chimeras were immunized with myelin oligodendrocyte glycoprotein (MOG_{35-55}) as described previously (Gyulveszi et al.) and CNS-invading T cells were analyzed for their secretion of pro-inflammatory cytokines such as GM-CSF, IL-17A and IFN-γ. Figure 1A shows that T cells unresponsive to IL-12 alone produced elevated levels of GM-CSF compared to wt T cells, whereas the combined loss of IL-12 and IL-23 responsiveness renders T cells unable to secrete GM-CSF **(Fig1A)** and IL-17A (Gyulveszi et al., 2009).

Mice lacking IL-12 (p35) or IFN-γ have been described to be hyper-susceptible to EAE (Becher et al., 2002b; Chu et al., 2000a; Cua et al., 2003c) and it was claimed that this is due to an increased secretion of IL-17A in the absence of T_H1 cytokines (Harrington et al., 2005b). However, as discussed above, IL-17A has a redundant function in the development of EAE and thus, we wanted to determine the influence of T_H1 cytokines on the polarization of GM-CSF producing T_H cells. We stimulated splenocytes obtained from MOG_{35-55}-specific TCR transgenic mice (2D2) in the presence of their cognate antigen, together with anti-CD28 antibody, while blocking endogenous IL-12 and IFN-γ. We found that inhibiting IL-12 as well as IFN-γ drastically enhanced GM-CSF secretion by MOG_{35-55} -specific T_H cells **(Fig.1B)**. To verify that IL-12 and IFN-γ are indeed negative regulators of GM-CSF production, we isolated lymphocytes from IL-12Rβ2$^{-/-}$ and IFN-γ$^{-/-}$ mice and showed again that they produce significantly higher levels of GM-CSF after polyclonal stimulation when compared to wt mice (**Suppl.Fig1**).

To ascertain that GM-CSF and not IL-17 or IFN-γ secretion is the driving force behind the pathogenicity of autoimmune T_H cells, we isolated splenocytes from MOG_{35-55} -immunized 2D2 (CD45.1 congenic) mice and cultured them under conditions which favored the generation of either GM-CSF, IL-17 or IFN-γ production. After 3 days of culture, $6x10^6$ cells were adoptively transferred into wt mice in order to evaluate their encephalitogenic potential. While all three populations were able to initiate the development of EAE, recipients of GM-CSF producing T_H cells had a significantly earlier onset of the disease and a drastically increased disease severity (**Fig.2A**). MOG-reactive T cells were analyzed prior to transfer and isolated from the CNS at different time points during disease (**Fig.2B and C**). Most of the infiltrating MOG_{35-55} -specific T cells retained the secretion of GM-CSF when initially stimulated under GM-CSF polarizing conditions (i.e. blockade of IL-12 and IFN-γ). Interestingly, the CNS-invading T cells of mice receiving T_H17 and T_H1 cells do acquire the capacity to secrete GM-CSF simultaneously. This finding supports the idea that regardless of the *in vitro* polarizing conditions, it is the ability of T cells to secrete GM-CSF, which makes them encephalitogenic. The plasticity of T cell cytokine secretion *in vivo* is not surprising and T_H17 cells were already demonstrated to not represent a terminally differentiated stable cell lineage (Zhou et al., 2009a). On the other hand, T_H1 cells were initially only mildly encephalitogenic, but GM-CSF secretion of CNS-invading T cells correlated with disease progression (**Fig.2C**).

Next, we purified the polarized cells based on their cytokine profile prior to adoptive transfer. Although the intracellular staining allows the functional study of the population of interest, it requires the fixation and permeabilization of the cell membrane, which inevitably causes cell death. For this reason we extended a method that was developed to capture the freshly released cytokine of interest on the surface of secreting cells. Instead of transferring the entire polarized MOG_{35-55}-reactive T cell pool, we enriched $CD4^+GM\text{-}CSF^+$, $CD4^+IL\text{-}17A^+$ and $CD4^+IFN\text{-}\gamma^+$ T cells respectively by FACS-sorting prior to adoptively transferring them into recipient wt mice (**Fig.3A**). Mice receiving only $6x10^5$ purified $CD4^+GM\text{-}CSF^+$ T cells developed disease with an early onset and high severity, while the same number of $CD4^+IL\text{-}17A^+$ and $CD4^+IFN\text{-}\gamma^+$ cells induced a significantly milder clinical score (**Fig. 3B**). Again, cells isolated from the CNS of afflicted animals on day 21 and analyzed, based on the congenic marker, on the one hand retained their original polarization pattern while on the other hand co-expressed GM-CSF, confirming the plasticity of inflammatory T_H cells *in vivo* (**Fig. 3C**).

To definitely demonstrate that GM-CSF is a crucial encephalitogenic T_H cytokine regardless of the polarization pattern initially induced, we used single cytokine deficient mice unable to secrete either GM-CSF or IL-17A crossed with MOG_{35-55} -TCR Tg mice (2D2). We immunized 2D2, 2D2xGM-$CSF^{-/-}$ and 2D2xIL-$17A^{-/-}$ mice, isolated and cultured lymphocytes in the presence of MOG_{35-55} peptide and IL-23 prior to transferring $5x10^6$ into wt mice. In line with the notion that GM-CSF is a mandatory T cell-derived factor in EAE, we found that 2D2 T cells or IL-$17A^{-/-}$-2D2 T cells induced EAE with similar kinetics. In contrast, 2D2xGM-$CSF^{-/-}$ T cells were incapable of inducing EAE (**Fig. 4A**). Fig. 4B depicts the lack of CNS-invading T cells in mice receiving 2D2xGM-$CSF^{-/-}$ T cells based on the congenic CD45.1 marker when compared with mice receiving 2D2 or 2D2xIL-$17A^{-/-}$ T cells (upper panel). Again, CNS-infiltrating 2D2 and 2D2xIL-$17A^{-/-}$ T cells produce GM-CSF indicating that GM-CSF-secretion is an absolute requirement for encephalitogenicity (**Fig. 4B** lower panel). 2D2 T cells lacking IFN-γ also expectedly induced EAE (data not shown). It is of note that 2D2xGM-$CSF^{-/-}$ T_H cells secrete high levels of IL-17A and IFN-γ when compared to 2D2 mice (**Suppl. Fig.2**). This finding underlines the fact that IL-17A secretion is not a functional feature required to induce neuro-inflammation.

T_H17 and T_H1 cells have been described to be maintained by the action of the transcription factors RORγt and T-bet, respectively. Both Rorc and T-bet deficient mice were shown to be EAE-resistant or to develop only mild disease symptoms (Bettelli et al., 2004; Ivanov et al., 2006). Loss of T-bet however was shown to not only inhibit T_H1 development, but to also severely impact the capacity of DCs to prime antigen-specific T cells (Lugo-Villarino et al., 2003). On the other hand, Rorc-deficient mice lack lymph nodes as well as lymphoid tissue inducer cells indicating that the reason for their EAE resistance lies not only in the polarization defect of T_H17 cells. To characterize GM-CSF secreting T_H cells at the molecular level we tested *in vitro* differentiated cells from naïve 2D2 mice for the expression of the transcription factors RORγt and T-bet. RORγt expression could not be observed in cells that did not receive any stimuli and in cells that were stimulated with MOG_{35-55} together with α-CD28 (**Fig.4C**). Strikingly, most of the cells cultured in the presence of blocking antibodies against IFN-γ and IL-12 expressed RORγt (**Fig.4C**). Also, as expected, most of the T cells cells differentiated in IL-17 skewing conditions expressed RORγt, while cells stimulated in T_H1 polarizing conditions expressed T-bet and not RORγt (**Suppl. Fig.3 and Fig.4C**). To assess if RORγt is in fact also required for the polarization of GM-CSF secreting T_H cells, we isolated lymphocytes of mice lacking Rorc ($Rorc^{GFP/GFP}$) mice and polarized them as described towards GM-CSF, IL-17 or IFN-γ secretion. The Rorc-deficient cells produce minimal quantities of GM-CSF even under GM-CSF skewing conditions indicating a dependency on RORγt expression for the differentiation of GM-CSF secreting pathogenic T_H cells. These cells also secreted small amounts of IL-17A but elevated levels of IFN-γ when compared to wt mice (**Fig.4D.**). Moreover, recently IL-27 has been shown to have a repressive function on RORγt as well as IL-17 expression (Diveu et al., 2009). This coincides with the increased EAE-susceptibility of $IL\text{-}27^{-/-}$ mice (Diveu et al., 2009). By adding IL-27 *in vitro* we not only observed a drastic reduction in IL-17A production but also a suppression of GM-CSF (**Suppl. Fig.4**)

In this report we demonstrate that GM-CSF secretion by T_H cells correlates exquisitely with the pathogenic potential of auto-aggressive T_H cells. The notion that GM-CSF may be the only known T cell-derived cytokine, which has a non-redundant role in CNS autoimmunity, integrates a number of phenomena related to EAE resistance and susceptibility reported over the past 20 years. IL-12, IFN-γ as well as IL-27 deficient mouse strains have been demonstrated consistently to be hyper-susceptible to EAE (Becher et al.; Cua et al.; Diveu et al.). Here we show that IL-12, IL-27 and IFN-γ inhibit the secretion of GM-CSF by T_H cells. In addition, *in vivo*, T_H cells lacking the ability to recognize IL-23 fail to invade the CNS (Gyulveszi et al.) and do not produce GM-CSF indicating that IL-23 is a positive regulator of GM-CSF expression by encephalitogenic T_H cells. GM-CSF production is dependent on the activity of RORγt and in contrast to IL-17A and IFN-γ, the loss of GM-CSF can be causally linked to the relative EAE resistance of $Rorc^{-/-}$ mice. None of the described T_H1 and T_H17 cytokines (IFN-γ, TNFα, IL-17A, IL-17F, IL-22, IL-21) has been shown to be mandatory for the pathogenic potential of myelin-reactive T_H cells (Chu et al.; Haak et al.; Hofstetter et al.; Kreymborg et al.; Sonderegger et al.). Using a series of adoptive transfer experiments we demonstrated that GM-CSF expression but neither IFN-γ nor IL-17 is critical for autoimmune T_H cells to induce EAE.

Given the lack of long-term stability of the T_H17 phenotype (Zhou et al., 2009a), we do not propose to name GM-CSF producing T cells T_H-GM-CSF cells. As one would expect from a responsive and plastic immune system, it is likely that GM-CSF similar to IL-17A, IL-17F, IL-9 or IL-22 is produced by T_H cells under particular circumstances in response to specific stimuli and a set of instructions by the local microenvironment. It is doubtful that the expression of these inflammatory cytokines is forced irreversibly upon a specific T cell “lineage”, at least *in vivo*. The precise means by which GM-CSF mediates encephalitogenicity remains to be resolved, but there is a large body of evidence, which indicates that GM-CSF is primarily an inflammatory cytokine rather than a growth factor (Hamilton, 2008a), which causes the expansion and activation of macrophages and DCs and within the CNS activates microglia (King et al., 2009; Ponomarev et al.). However, studies in BM-chimeras using GM-CSF receptor deficient mice indicate that GM-CSF activates CNS-invading rather than resident phagocytes (Heske et al. manuscript in preparation). In regards to MS, elevated levels of GM-CSF have been shown to correlate with the active phase of the disease in patients and administration of recombinant GM-CSF for hematopoietic progenitor cell mobilization leads to a disease exacerbations in MS patients (Openshaw et al.). These clinical data complemented our findings in the mouse model and highlight GM-CSF as a potential drug-target. The role of GM-CSF in MS will thus need to be investigated further. Taken together, we propose that GM-CSF rather than IL-17A or IFN-γ is the encephalitogenic factor produced by pathogenic T_H cells which resolves and integrates virtually all conflicting results regarding the function of RORc, IL-12, IFN-γ, IL-23 and IL-27 mutants and their phenotype during EAE.

METHODS SUMMARY

Mice

C57BL/6 (CD45.2), IL-12Rβ1$^{-/-}$, IL-12Rβ2$^{-/-}$, IFN-γ$^{-/-}$, GM-CSF$^{-/-}$ and RORc $^{GFP/GFP}$ mice were purchased from Jackson laboratories (Bar Harbour, Maine). MOG_{35-55} –specific TCR Tg mice (2D2) and IL-17A$^{-/-}$ mice were provided by V. Kuchroo (Harvard) and by Y. Iwakura (University of Tokyo). Animal experiments were approved by the Swiss Veterinary Office and performed according to federal and institutional guidelines. Bone marrow-chimeras were generated as described previously (Gyulveszi et al.).

T cell polarization

For the generation of GM-CSF or IL-17A or IFN-γ secreting T_H cells, splenocytes were isolated from naïve or MOG_{35-55}-immunized 2D2, wt, IFN-γ$^{-/-}$, IL-12Rβ2$^{-/-}$ or RORc $^{GFP/GFP}$ mice. Cells were cultured and stimulated with $MOG_{35–55}$ (Genscript) or anti-CD3 (2C11, Bioexpress, Lebanon) and anti-CD28 (37N, Bioexpress) antibodies plus different cytokines and neutralizing antibodies depending on the desired functional signature. Three days later cells were analyzed by intracellular staining or used for surface capture. IL-27 was obtained from R&D Systems (Minneapolis, MN) and added at day 0 together with the stimuli. For the in vitro generation of encephalitogenic T_H cells, splenocytes from MOG_{35-55} immunized 2D2 (GM-CSF$^{-/-}$, IL-17A$^{-/-}$, IFN-γ$^{-/-}$), mice were isolated at 7dpi and cultured in the presence of $MOG_{35–55}$ and 20ng/ml IL-23 (eBioscience, San Diego, CA) for 2 days before transfer.

FACS analysis and sorting

Extraction and staining of mononucleated cells from inflamed CNS tissue was performed as described previously (Gyulveszi et al.). For intracellular stainings cell preparations were fixed and permeabilized with fixation/permeabilization buffers (eBioscience) for transcription factor detection or with Cytofix/Cytoperm Plus Kit (BD Bioscience) for cytokine assay. The surface capture of cytokines is described elsewhere (Streeck et al., 2008). All flow cytometric analyses and cell sorting were performed respectively on a FACSCantoII (BD, Becton Dickinson Systems, Franklin, NJ) and on a FACSAria (BD).

Induction of EAE

Mice were immunized subcutaneously and clinical disease was observed as described previously (Greter et al., 2005a). For adoptive transfer experiments, splenocytes were isolated, cultured and $5x10^6$ bulk cells were injected as described previously (Stromnes and Goverman, 2006). In case enriched cytokine-positive T_H cells were adoptively transferred we injected only $6x10^5$ cells per wt mouse.

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INTEREST DECLARATION

The authors declare no conflicts of interest

FIGURE LEGENDS

Fig.1. **The capacity of autoimmune T cells to produce GM-CSF is dependent on IL-12Rβ1 rather than IL-12Rβ2 and inhibited by IL-12 and IFN-γ**

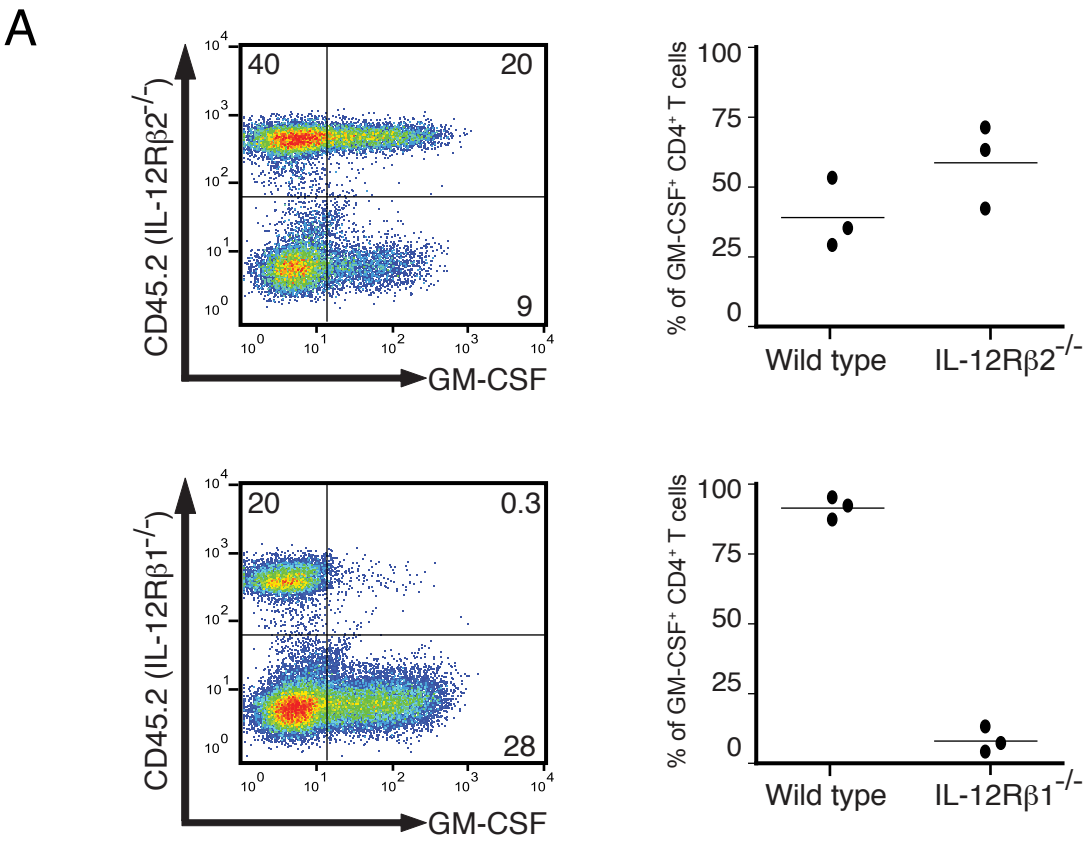
(A) EAE was induced in mixed BM-chimeric mice (n≥6) with MOG_{35-55}/CFA. 18 dpi GM-CSF production by CNS-infiltrating T_H cells was analyzed by flow cytometry in chimeric $RAG1^{-/-}$ mice reconstituted either with a 1:1 mixture of IL-12Rβ2$^{-/-}$ and wt (upper panel) or IL-12Rβ1$^{-/-}$ and wt BM marrow (lower panel). The scatter plots on the right show the percentages of GM-CSF producing cells in three independent experiments. (B) Splenocytes obtained from TCR Tg 2D2 mice were isolated and stimulated for 72h with MOG_{35-55} and α-CD28 together with either IL-12 and IFNγ or mAbs agains IL-12 and IFNγ. $CD4^+$ T cells were then analyzed by flow cytometry for the expression of GM-CSF and IFN-γ. Shown is a representative of at least 5 independent experiments.

Fig.2. **GM-CSF secreting autoimmune T cells are highly encephalitogenic** (A) TCR Tg 2D2 cells were polarized to secrete GM-CSF, IL-17A or IFN-γ prior to their adoptive transfer into wt mice. The animals were observed for clinical disease development. (B) The cytokine profile of the polarized MOG_{35-55} reactive T cells was analyzed by flow cytometry prior adoptive transfer after 3 days in culture. (C) IFN-γ, IL-17A and GM-CSF production of CNS- infiltrating $CD4^+$ T cells after 13 and 17 days post cell transfer (gated on CD4).

Fig.3. **Enriched GM-CSF producing cells have a superior pathogenic capacity compared to IFN-γ or IL-17A polarized T_H cells.** (A) TCR Tg 2D2 cells were polarized as described for 3 days *in vitro* and subsequently enriched using cytokine cell-surface capture. Depicted are the populations after FACS sorting. (B) Clinical disease development in wt after adoptive transfer of $6x10^5$ of enriched cytokine-secreting cells (C) CNS infiltrating 2D2 CD4 T cells were analysed for cytokine production at day 21 post transfer (gated on CD4 and CD45.1). Shown is a representative of at least 3 independent experiments.

Fig.4. GM-CSF-secretion is essential for the pathogenicity of autoimmune T cells and dependent on RORγt expression. (A) 2D2 mice were crossed with IL-17A$^{-/-}$ and GM-CSF$^{-/-}$ mice. Lymphocytes were isolated and activated with MOG_{35-55} and IL-23 prior to adoptive transfer and clinical disease development was observed. (B) At peak disease (day 19 post transfer), CNS-invading T cells were quantified based on CD45.1 expression and CD11b (upper panel). The lower panel shows the secretion of IL-17A and GM-CSF by CNS –invading 2D2 T cells (Gated on CD45.1 and CD4) (C) RORγt expression in different *in vitro* polarized CD4 T cell subsets (D) GM-CSF profile of wt and Rorc $^{GFP/GFP}$ CD4 T cells under different *in vitro* skewing conditions.

Fig.1


A
CD45.2 (IL-12Rβ2-/-)
GM-CSF
40
20
9
% of GM-CSF+ CD4+ T cells
Wild type
IL-12Rβ2-/-
CD45.2 (IL-12Rβ1-/-)
20
0.3
28
Wild type
IL-12Rβ1-/-

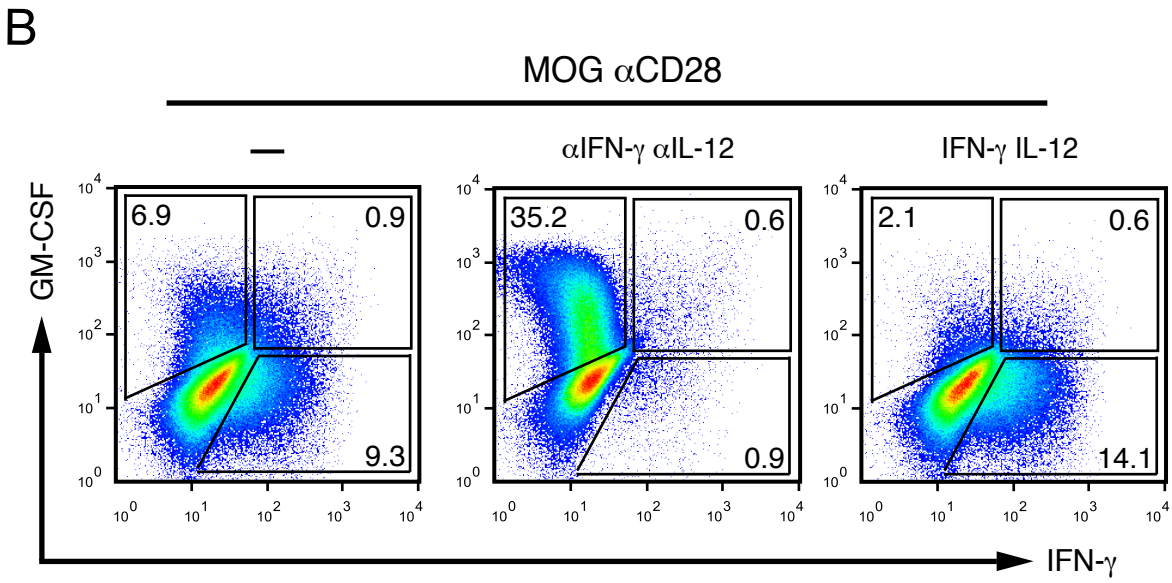

B
MOG αCD28
—
αIFN-γ αIL-12
IFN-γ IL-12
GM-CSF
IFN-γ
6.9
0.9
9.3
35.2
0.6
0.9
2.1
0.6
14.1

Fig.2

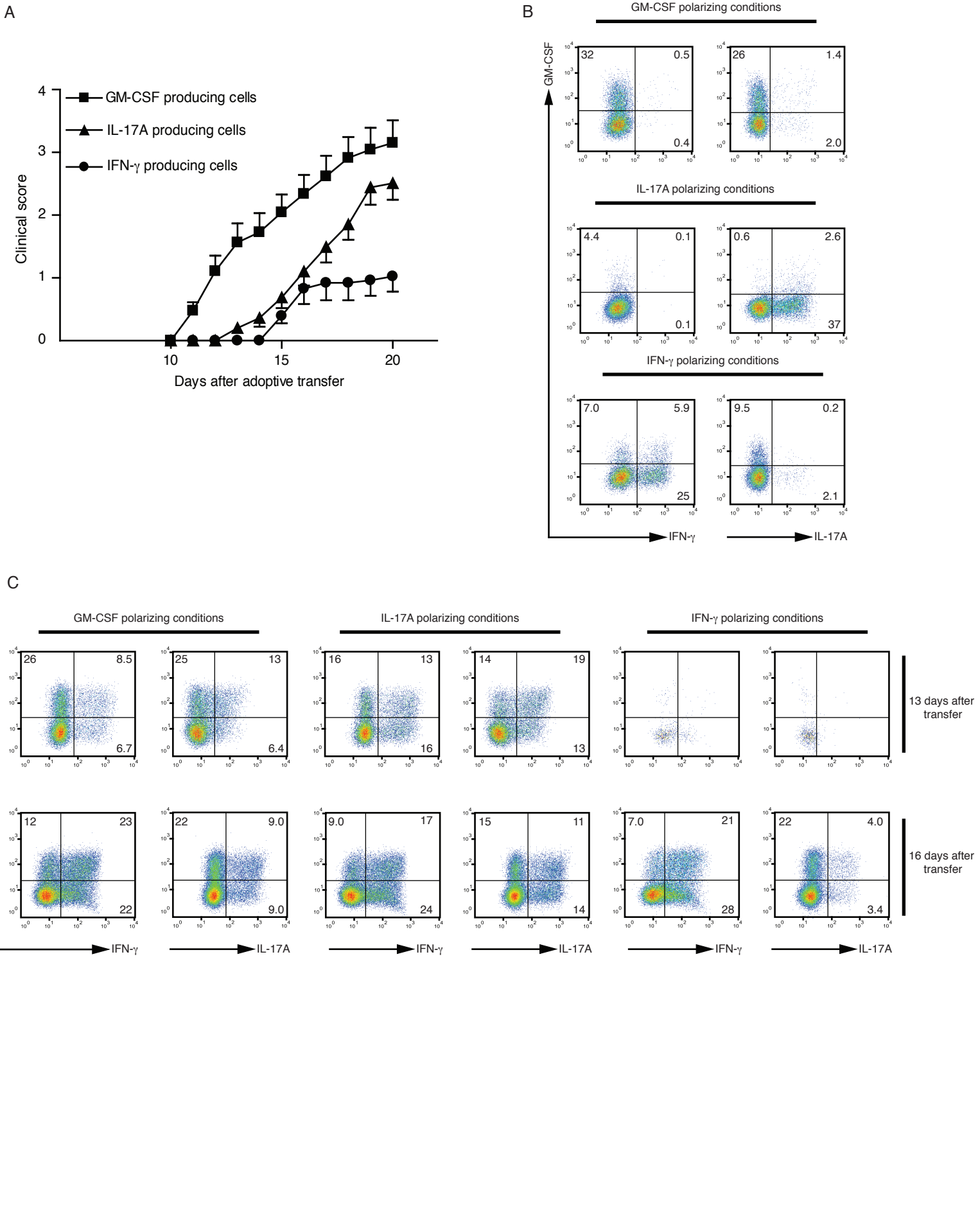
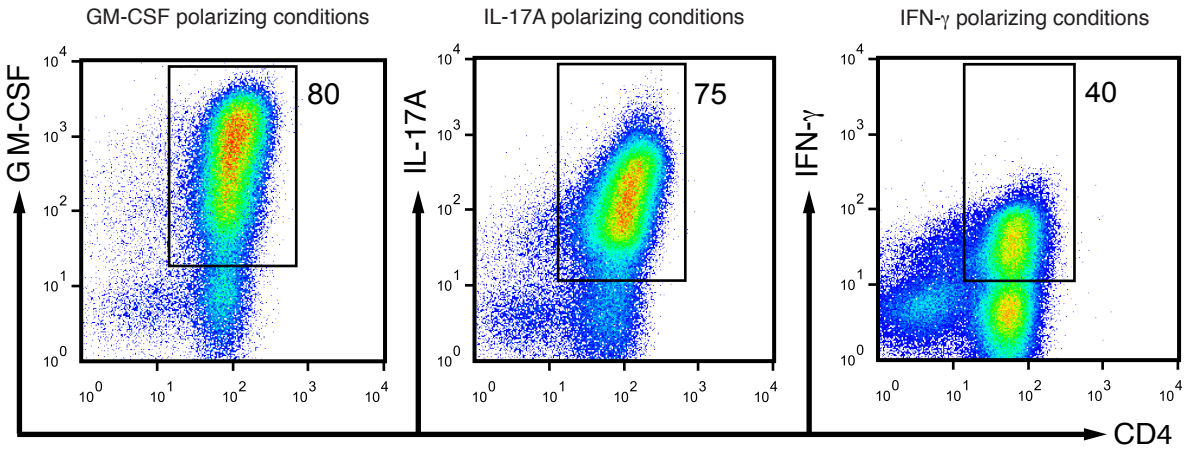

A
GM-CSF producing cells
IL-17A producing cells
IFN-γ producing cells
Clinical score
Days after adoptive transfer
B
GM-CSF polarizing conditions
IL-17A polarizing conditions
IFN-γ polarizing conditions
GM-CSF
IFN-γ
IL-17A
C
GM-CSF polarizing conditions
IL-17A polarizing conditions
IFN-γ polarizing conditions
13 days after transfer
16 days after transfer
GM-CSF
IFN-γ
IL-17A

Fig.3

A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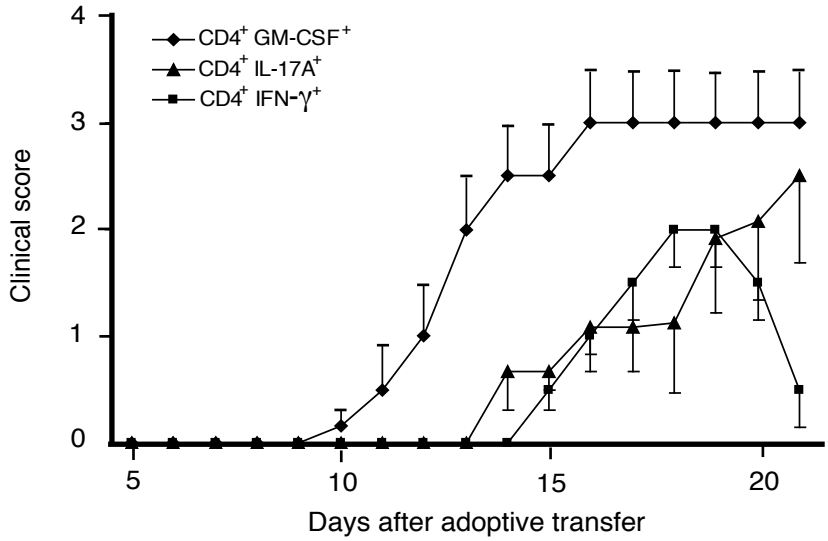
IL-17A polarizing conditions
IFN-γ polarizing conditions
GM-CSF
IL-17A
IFN-γ
80
75
40
CD4

B


CD4+ GM-CSF+
CD4+ IL-17A+
CD4+ IFN-γ+
Clinical score
Days after adoptive transfer

C

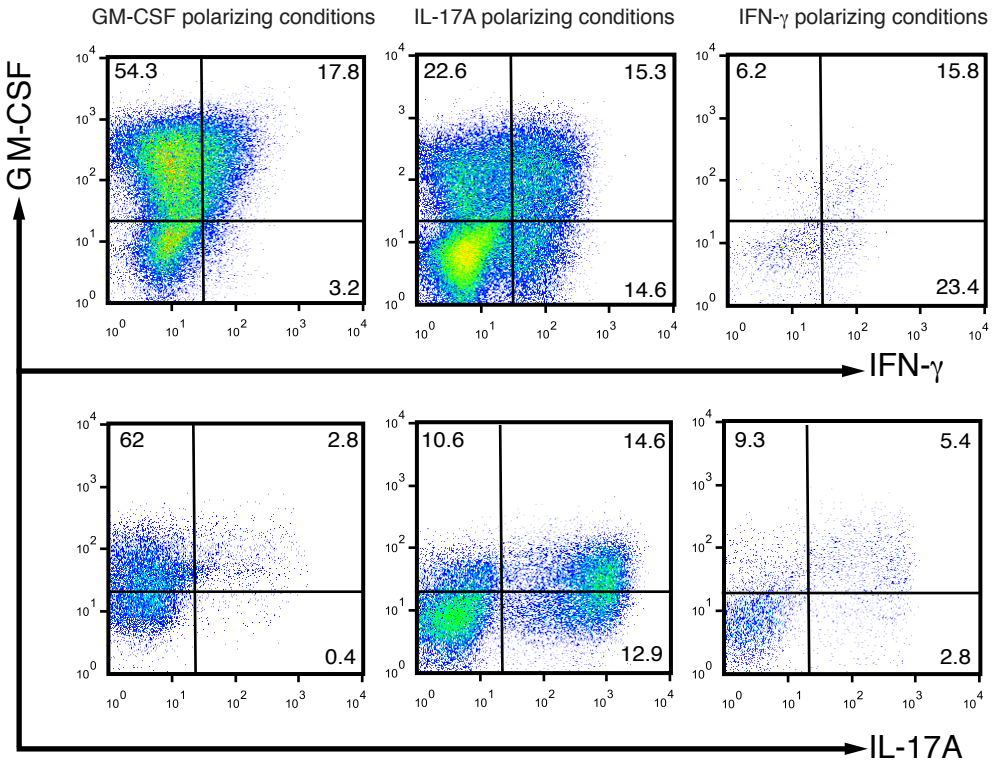
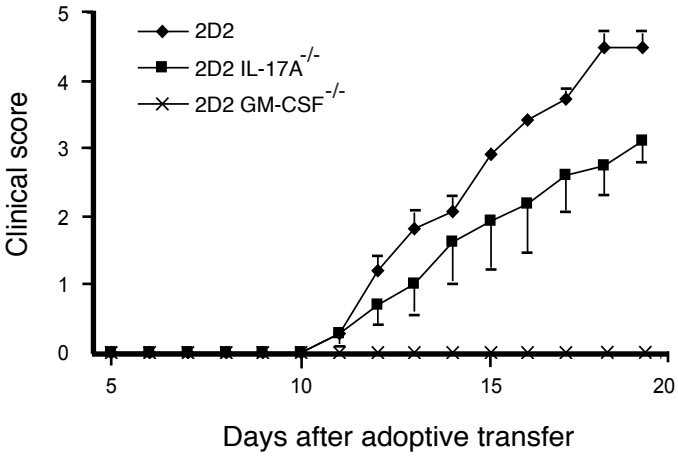
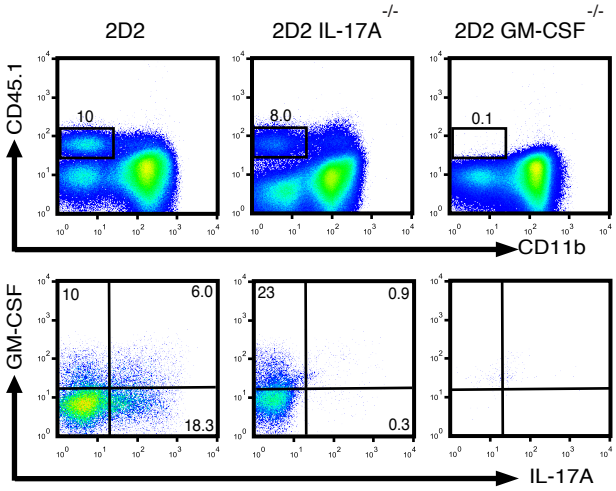

GM-CSF polarizing conditions
IL-17A polarizing conditions
IFN-γ polarizing conditions
GM-CSF
IFN-γ
54.3
17.8
3.2
22.6
15.3
14.6
6.2
15.8
23.4
IL-17A
62
2.8
0.4
10.6
14.6
12.9
9.3
5.4
2.8

Fig.4

A


Clinical score
Days after adoptive transfer
2D2
2D2 IL-17A-/-
2D2 GM-CSF-/-

B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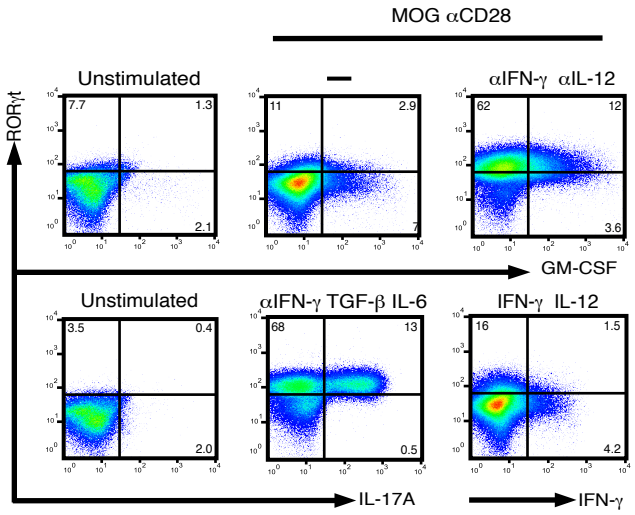
C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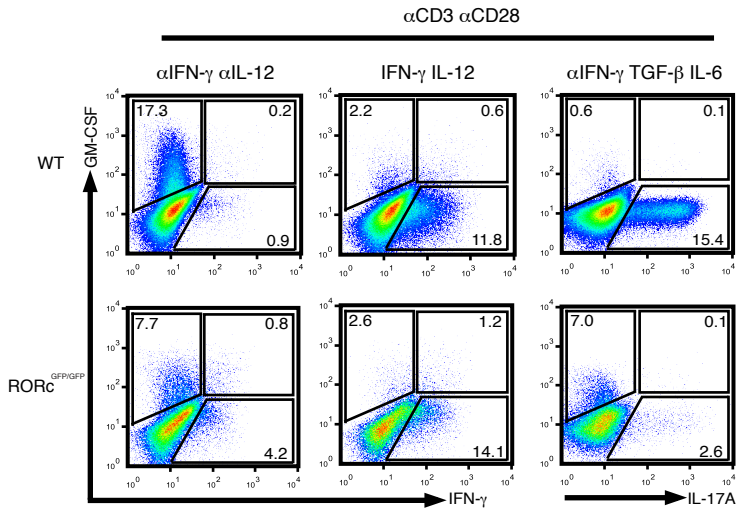
IFN-γ

D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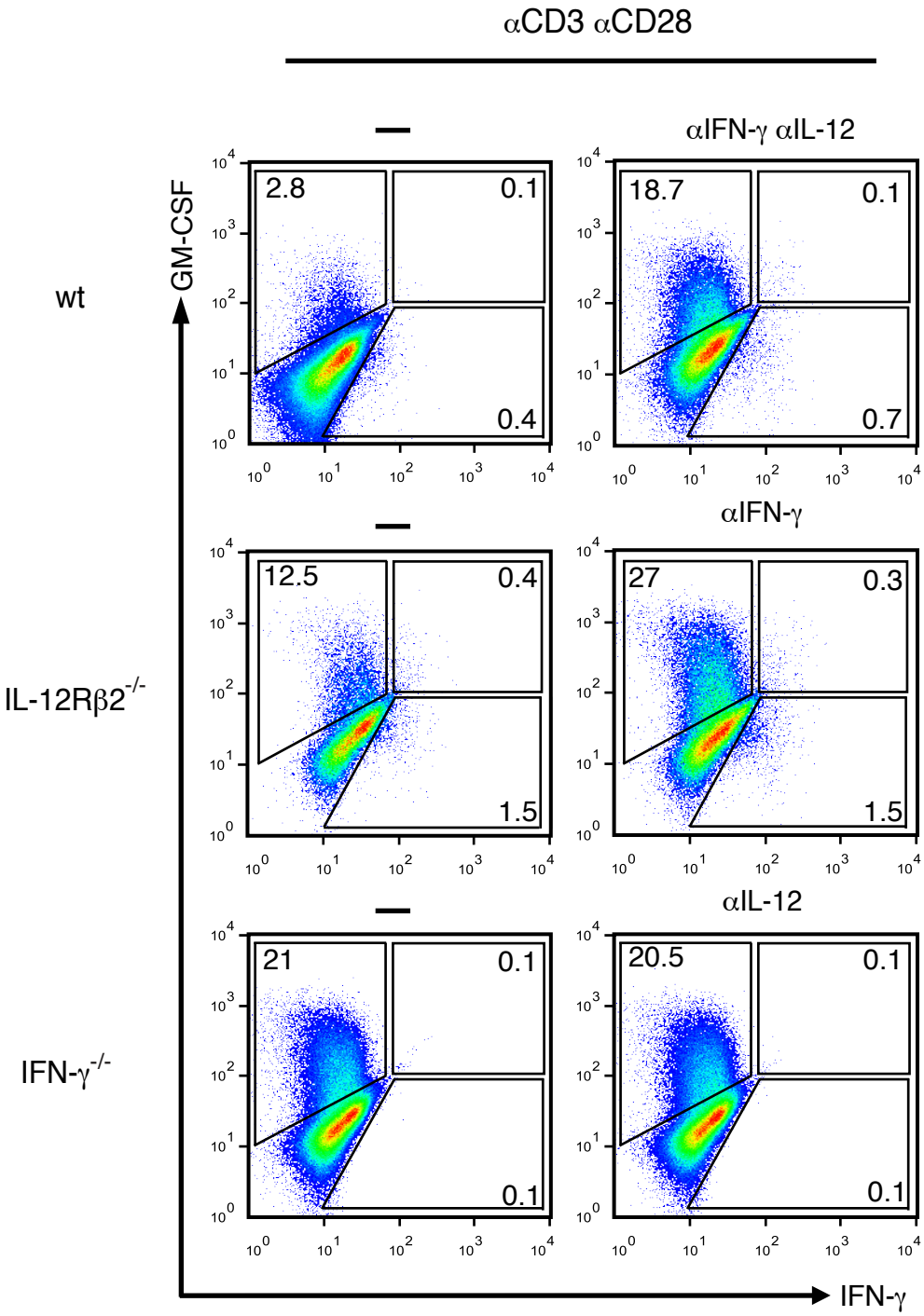
IL-17A

Suppl. Fig1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0.4
18.7
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0.7
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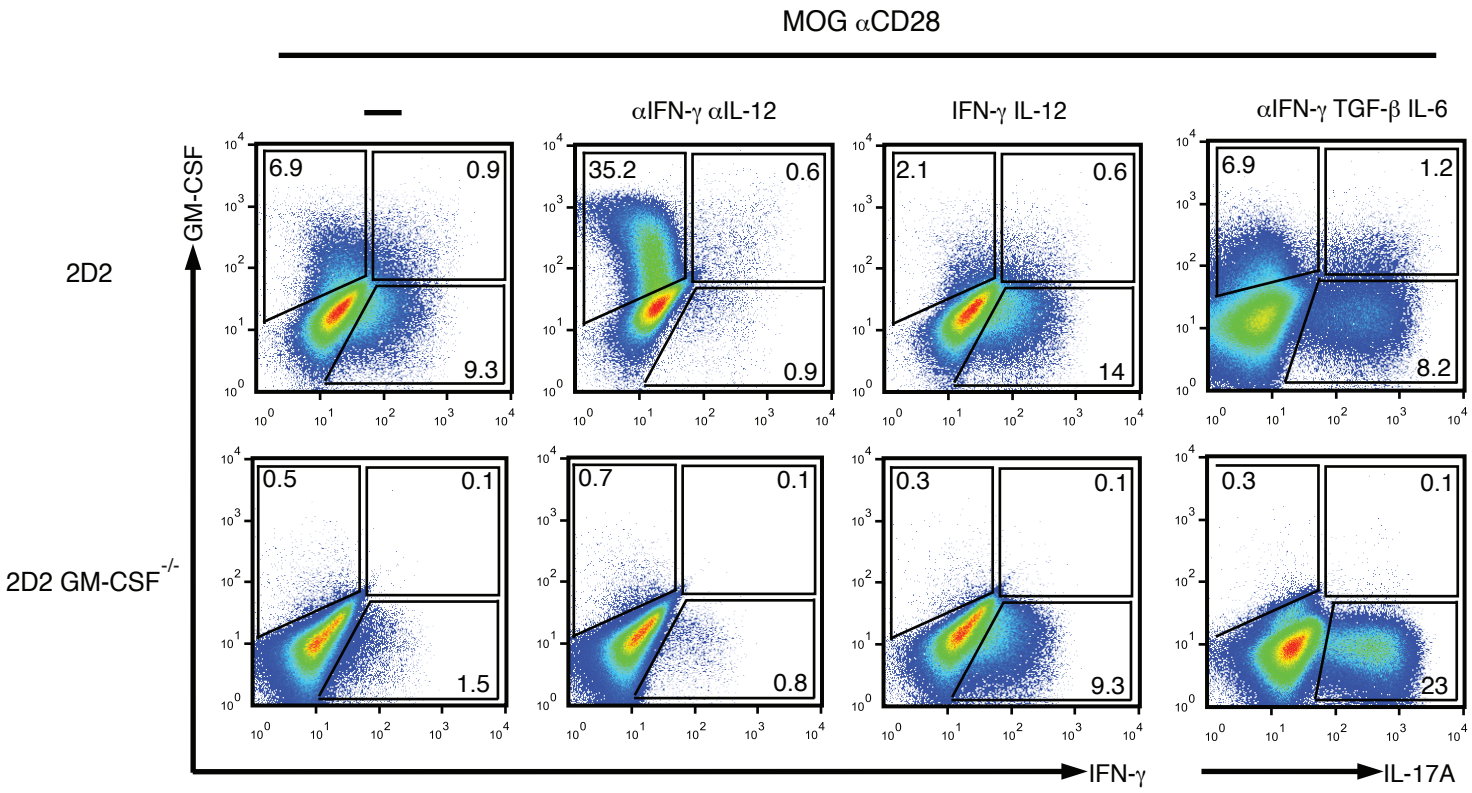
Suppl. Fig.2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IFN-γ
IL-17A
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0.9
9.3
35.2
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0.9
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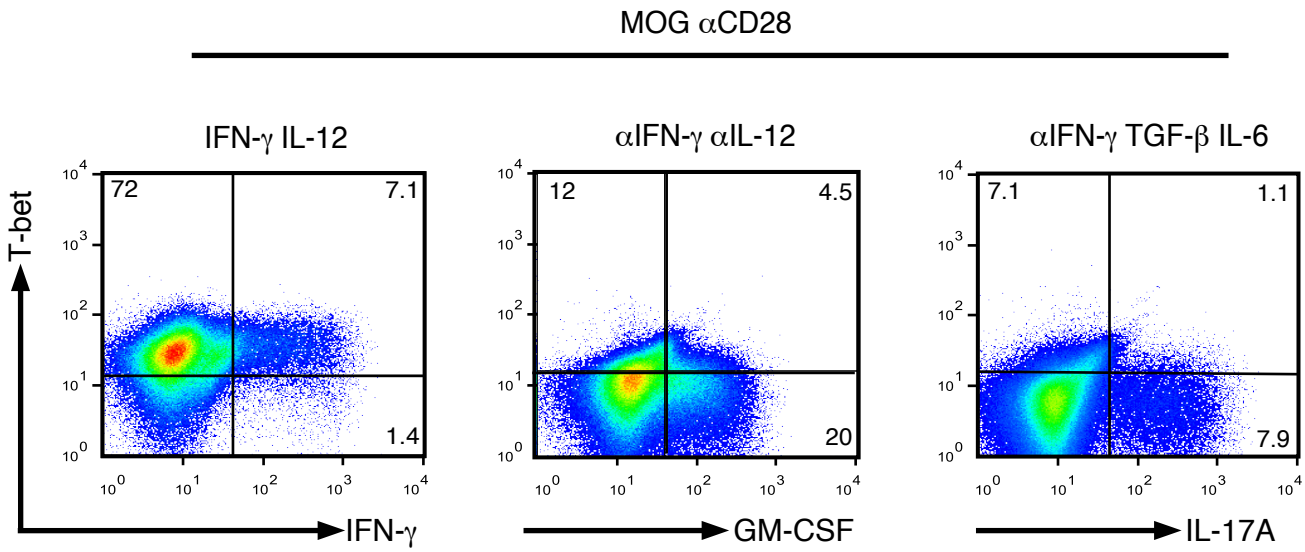
Suppl. Fig.3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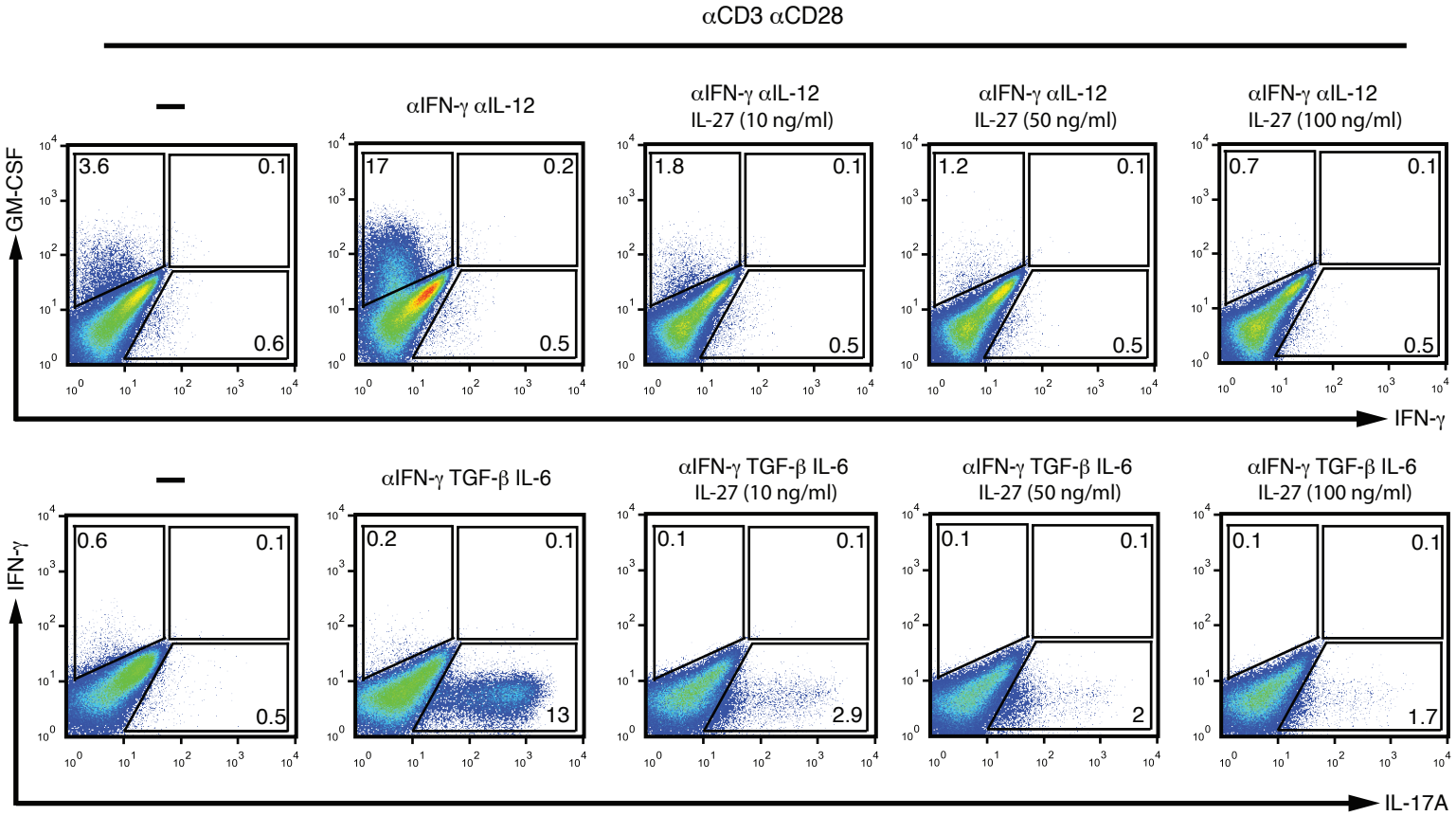
7.1
1.1
7.9

Suppl. Fig.4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αIFN-γ αIL-12
IL-27 (100 ng/ml)
GM-CSF
IFN-γ
3.6
0.1
0.6
17
0.2
0.5
1.8
0.1
0.5
1.2
0.1
0.5
0.7
0.1
0.5
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αIFN-γ TGF-β IL-6
αIFN-γ TGF-β IL-6
IL-27 (10 ng/ml)
αIFN-γ TGF-β IL-6
IL-27 (50 ng/ml)
αIFN-γ TGF-β IL-6
IL-27 (100 ng/ml)
IFN-γ
IL-17A
0.6
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2
0.1
0.1
1.7

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METHODS (online)

Mice

C57BL/6 (CD45.2, also referred to as wt), IL-12Rβ1$^{-/-}$, IL-12Rβ2$^{-/-}$, IFN-γ$^{-/-}$, GM-CSF$^{-/-}$ and RORc $^{GFP/GFP}$ mice were purchased from Jackson laboratories (Bar Harbour, Maine) and bred in house under specific pathogen-free conditions. MOG_{35-55} –specific TCR Tg mice (2D2) were generously provided by V. Kuchroo (Harvard) and IL-17A$^{-/-}$ mice were provided by Y. Iwakura (University of Tokyo). Animal experiments were approved by the Swiss Veterinary Office (13/2006; Zurich, Switzerland) and performed according to federal and institutional guidelines.

To generate bone marrow-chimeras, RAG1$^{-/-}$ mice were lethally irradiated with a split-dose of 1100 rad (Gyulveszi et al.). Donor animals were euthanized with CO_2 and bones (fore- and hind-legs, hips) were flushed with sterile PBS to obtain bone marrow stem cells. Mutant and wt BM with the respective congenic CD45 marker were mixed at a 1:1 ratio. The mixture was tested by flow cytometry with anti-CD45.1 (A20) and anti-CD45.2 (104) mAb (BD Pharmingen). In total, $2x10^7$ cells were injected i.v. per mouse and to prevent bacterial infection 0.2% BORGAL was added for 2 weeks to the drinking water.

T cell polarization

For the generation of GM-CSF or IL-17A or IFN-γ secreting T_H cells, splenocytes were isolated from naïve or MOG_{35-55}-immunized 2D2, wt, IFN-γ$^{-/-}$, IL-12Rβ2$^{-/-}$ or RORc $^{GFP/GFP}$ mice. Cells were cultured and stimulated with 20 µg/ml $MOG_{35–55}$ (Genscript) or 5 µg/ml anti-CD3 (2C11, Bioexpress, Lebanon, NH) and 5 µg/ml anti-CD28 (37N, Bioexpress) antibodies plus different cytokines and neutralizing antibodies depending on the desired functional signature: 10 µg/ml aIFN-γ (R4-6A2; Bioexpress, Lebanon) and 10 µg/ml aIL-12 (Bioexpress), to induce GM-CSF secreting cells, 10 µg/ml aIFN-γ together with 10 ng/ml TGF-β and 20 ng/ml IL-6 (both from PeproTech, Rocky Hill, NJ) to differentiate T_H17 cells, and 10 ng/ml of IFN-γ and 5 ng/ml of IL-12 (both from PeproTech) to generate T_H1 cells. Cells were analyzed on day 3 post-seeding by intracellular staining or used for surface capture and purified on the base of their cytokine expression. IL-27 was obtained from R&D Systems (Minneapolis, MN) and added at day 0 together with the stimuli. For the in vitro generation of encephalitogenic T_H cells, splenocytes from MOG_{35-55} immunized 2D2 (GM-CSF$^{-/-}$, IL-17A$^{-/-}$, IFN-γ$^{-/-}$), mice were isolated at 7dpi and cultured in presence of 20 µg/ml $MOG_{35–55}$ and 20 ng/ml IL-23 (eBioscience) for 2 days and then adoptively transferred into wt animals as described (Stromnes and Goverman, 2006).

FACS analysis and sorting

Extraction and staining of mononucleated cells from inflamed CNS tissue was performed as described previously (Gyulveszi et al.). For intracellular RORγt and T-bet analysis, cell preparations were fixed and permeabilized with fixation/permeabilization buffers (eBioscience, San Diego, CA) after staining of cell surface markers and stained with PE or APC-conjugated rat α-RORγt (AFKJS-9, eBioscience) or APC –conjugated αT-bet antibodies (4-B10, eBioscience). For the surface capture of cytokines (Streeck et al., 2008) cells were incubated after 3 days of *in vitro* culture with a biotinilated antibody specific for either GM-CSF (MP1-31G6, eBioscience), IL-17A (TC11-8H4, eBioscience) or IFN-γ (R4-6A2, eBioscience) linked through an avidin (Invitrogen, Carlsbad, CA) bridge to an anti-CD3 (145-2C11, eBioscience) antibody before being re-stimulated for 3 hours with PMA (50 ng/ml) and Ionomycin (500 ng/ml). The cells were then stained on the surface with APC-Cy7 α-CD4 antibody and PE α-GM-CSF (MP1-22E9, eBioscience), PE α-IL-17A (TC11-18H10, BD Pharmingen) or FITC α-IFN-γ (XMG1.2, BD Pharmingen) antibody and sorted based on their functional profile. All flow cytometric analyses were performed on a FACSCantoII (BD, Becton Dickinson Systems, Franklin, NJ), while cell sorting was performed on a FACSAria (BD).

For intracellular cytokine staining, the cells were stimulated for 6 hours with PMA (50 ng/ml) and Ionomycin (500 ng/ml) in the presence of Golgi plug (BD Pharmingen). Then they were stained for surface molecules, fixed/permeabilised with Cytofix/Cytoperm Plus Kit (BD Bioscience) and stained with the following antibodies: PE rat anti-mouse GM-CSF (MP1-22E9, BD Pharmingen), Alexa-647 rat anti–mouse IL-17A (eBioscience) and FITC rat anti-mouse IFN-γ (BD Pharmingen).

Induction of EAE

Mice were immunized subcutaneously with 200 µg of $MOG_{35–55}$-peptide emulsified in complete Freund's adjuvant supplemented with 2 mg/ml of *Mycobacterium tuberculosis* into the lateral abdomen and 200 µg of pertussis toxin in PBS was administered intra-peritoneally on day 0 and day 2. Clinical disease was observed as described (Greter et al., 2005a). For adoptive transfer experiments, 7 days after immunization, splenocytes were isolated and cultured, as described above, for 3 days under polarizing conditions. $5x10^6$ bulk cells were injected intra-peritoneally into sublethally irradiated mice (Stromnes and Goverman, 2006). In case enriched cytokine-positive T_H cells were adoptively transferred we injected only $6x10^5$ cells per wt mouse.

IL-23-driven encephalo-tropism and TH17 polarization during CNS-inflammation

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Abstract

IL-23 but not IL-12 is essential for the development of autoimmune tissue inflammation in mice. Conversely, IL-12 and IL-23 impact on the polarization of T_H1 and T_H17 cells respectively. While both polarized T helper populations can mediate autoimmune inflammation, their redundancy in the pathogenesis of EAE indicates that IL-23 exerts its crucial influence on the disease independent of its T helper polarizing capacity. To study the impact of IL-23 and IL-12 on the behavior of encephalitogenic T cells *in vivo*, we generated BM chimeric mice in which we can trace individual populations of IL-23 or IL-12 responsive T helper cells during EAE. We observed that T cells, which lack IL-12Rb1 (no IL-12 and IL-23 signaling), fail to invade the CNS and do not acquire a T_H17 phenotype. In contrast, loss of IL-12 signaling prevents T_H1 polarization but does not prevent T cell entry into the CNS. The loss of IL-12R engagement does not appear to alter T cell expansion but leads to their accumulation in secondary lymphoid organs. We found that IL-23 licenses T cells to invade the target tissue and to exert their effector function while IL-12 is critical for T_H1 differentiation, but does not influence the pathogenic capacity of auto-reactive T helper cells *in vivo*.

Introduction

EAE is an inflammatory disease of the CNS that is induced in susceptible animals by immunization with myelin proteins or peptides. It serves as a reliable animal model for CNS inflammation and autoimmunity[1]. The disease is the result of a $CD4^+$ T cell–mediated immune response directed at the myelin sheath within the CNS. For many years T_H1 cells were believed to be the most encephalitogenic population of T cells[2]; more recently T_H17 cells were claimed to have more pathogenic potential than T_H1 cells[3]. While the inflammatory signature and regulation of T_H17 cells has been studied in depth, a single key molecule produced by T_H17 cells and responsible for the encephalitogenic properties of T helper cells remains to be discovered[4;5]. Numerous studies had inadvertently indicated the pivotal role of the cytokine IL-12 in the pathogenesis of EAE, as eliminating either the IL-12Rb1 subunit of the receptor or the p40 subunit of the cytokine renders mice resistant to EAE[6]. However, unexpectedly the deletion of the second IL-12 subunit, p35 or the signaling subunit of the receptor, IL-12Rb2, has either no impact on disease susceptibility or results in an even more severe clinical outcome[7;8]. The discovery of IL-23 provided an explanation for the discrepancies observed in studies performed with p40 and p35 deficient animals as IL-12 and IL-23 share subunits and receptor components. IL-23 is a heterodimeric protein composed of two disulfide-linked subunits, p40, which is shared with IL-12 and a unique p19 subunit[9]. Similarly to IL-12, only activated accessory cells such as monocytes, macrophages and DCs concomitantly express both subunits to form the active cytokine[9]. Mice deficient for p19, can efficiently generate T_H1 cells but fail to develop EAE after immunization with MOG[3;10]. Taken together these observations, IL-23 and not IL-12 is a key player during the development of the autoimmune inflammation of the CNS. Initial studies suggested that IL-23 is involved in the generation of the newly described T_H17 cells. However, other groups have shown that IL-23 alone does not influence the *de novo* differentiation of naïve T cells into T_H17 cells *in vitro* and it is only efficient in inducing the proliferation of committed IL-17 producing effector and memory T cells[11;12]. Several groups have addressed the question as to which factors initiate T_H17 polarization in naïve T cells. In contrast to T_H1 and Th2 cells, the development of T_H17 cells is not dependent of its respective effector cytokine (IL-17A). Instead, IL-6 and TGF-β, two cytokines with generally opposing effects, were found to direct the *de novo* generation of T_H17 cells[11;13;14]. TGF-β induces the generation of Foxp3 expressing regulatory T cells, while addition of IL-6, a potent pro-inflammatory cytokine, deviates TGF-β driven regulatory T cell toward T_H17 polarization. Moreover, the expression of the transcription factor ROR-gt that has been shown to be critical for T_H17 lineage determination is induced by the combination of TGF-β and IL-6[15]. The mechanistic underpinnings of IL-23 function during clinical disease development and T_H17 polarization is not yet completely understood, although

some reports indicate that it is required for the survival and further expansion of already differentiated T_H17 effector cells[16-18].

The receptor for IL-23 is a heterodimer composed of IL-12Rb1 and a unique subunit, IL-23R[19], whereas the receptor for IL-12 consists of the shared IL-12b1 chain and the specific IL-12Rb2 chain[20]. Thus, cells lacking IL-12Rb1 cannot be engaged by either IL-12 or IL-23, while IL-12Rb2-deficient cells are unresponsive to IL-12 but still responsive to IL-23. To trace IL-12 and IL-23-dependent behavior of T cells during EAE, we generated mixed BM-chimeric mice and found that in contrast to purified naïve T cells *in vitro*, encephalitogenic T_H17 cell expansion *in vivo* is absolutely dependent on IL-23. Furthermore, loss of IL-23 signaling aborts the capacity of T cells to invade the CNS causing their accumulation in secondary lymphoid tissues.

Results and discussion

Construction of the model

IL-23 is a prerequisite for EAE development but its role under pathological conditions remains elusive, due to the EAE resistance of mice lacking IL-12Rb1$^{-/-}$, p40$^{-/-}$ and p19$^{-/-}$. In order to reveal IL-23-dependent T cell behavior during EAE, we generated bone-marrow chimeric mice **(Fig. 1A)**. RAG1$^{-/-}$ mice were reconstituted with a mixture of either wt and IL-12Rβ1$^{-/-}$ BM cells or wt and IL-12Rβ2$^{-/-}$ BM cells. The gene-targeted cell population in the former chimera (IL-12/23R KO) is not responsive to IL-12 and IL-23, while the latter (IL-12R KO) is responsive to IL-23, but not to IL-12. The wt component of the graft is responsive to both cytokines and capable of initiating EAE permitting us to trace the behavior of the mutant T cells during inflammation. Each cell population carries a traceable congenic marker (CD45.1 = wt vs CD45.2 = IL-12Rβ1$^{-/-}$ or IL-12Rβ2$^{-/-}$) on the surface. After a recovery period, peripheral blood of chimeric animals was analyzed and the quantification of mature cell populations reflected the ratio (approximately 1:1) observed in the donor BM grafts (data not shown).

To induce EAE, BM chimeras were injected with MOG_{35-55} peptide emulsified in CFA without an additional injection of pertussis toxin. First signs of EAE were observed at 12 dpi without significant differences between the two groups, in terms of disease incidence and disease severity **(Fig. 1B and C)** although IL-12R KO chimeras as expected showed a slightly more severe disease consistent with the hypersusceptibility of the IL-12Rb2$^{-/-}$ genotype. These mixed BM-chimeras allow the comparison of the behavior of wt and receptor deficient cells in the same diseased animal, which could not be studied in disease-resistant cytokine or cytokine receptor deficient mice.

IL-23 affects the CNS-tropism of CD4$^+$ T cells but not systemic expansion and activation

In order to investigate the role of IL-23 on T cells during EAE, CNS-infiltrating cells were isolated from BM-chimeras and were analyzed by flow cytometry. Consistent with disease severity, massive infiltration of mononuclear cells was observed within the CNS of both groups. In the IL-12R KO chimeras we found a 1:1 ratio of wt and IL-12Rβ2$^{-/-}$ CD4$^+$ T cells infiltrating the CNS **(Fig. 2A)**. In contrast, in the IL-12/23R KO chimeras we observed an overwhelming infiltration of IL-12/23 responsive wt CD4$^+$ T cells, while IL-12Rβ1$^{-/-}$ CD4$^+$ T cells failed to accumulate in the CNS **(Fig. 2B)**. Absolute numbers of CNS-infiltrating T cells are provided in **Supporting Information Figure 1A**. The failure to detect equal numbers of CNS-infiltrating IL-12Rβ1$^{-/-}$ CD4$^+$ T cells demonstrates the vital function of IL-23 in conferring encephalo-tropism of T cells, while the lack

of IL-12-mediated signaling did not influence the capacity of T cells to infiltrate the CNS during EAE. In accordance with a recent report by McGeachy et al, the IL-12Rβ1 mediated impact on T cell function is indeed mediated by IL-23R complex rather than additive effects of IL-23 and IL-12[21]. To analyze the role of IL-12/23 or IL-12 signaling alone in the systemic immune compartment, spleen and LNs cells were isolated at 16dpi for cytofluorometric analysis from MOG_{35-55} immunized BM-chimeras. As we observed previously, in the IL-12/23R KO chimeric animals where the number of IL-12Rβ1-deficient T cells infiltrating the CNS was drastically reduced, we detected their accumulation in both spleen and LNs when compared to wt $CD4^+$ T cells in the same animal **(Fig. 2C)**. At the same time in the IL-12R KO chimeric mice the ratio of peripheral IL-12Rβ2$^{-/-}$ $CD4^+$ T cells to wt $CD4^+$ T cells was unaffected **(Fig. 2D)**. This finding indicates that the lack of IL-23R engagement does not interfere with the expansion of autoreactive T cells, but with the capacity to leave secondary lymphoid organs and to access their target tissue. Absolute numbers of T cells accumulating in secondary lymphoid organs are provided in **Supporting Information Figure 1A.**

IL-12Rb1 signaling is essential for the secretion of IL-17 by T cells in vivo

To address how IL-12 and IL-23 affect T cell polarization *in vivo* during EAE, we analyzed the cytokine production of the infiltrating cell populations by intracellular cytokine staining and flow cytometry. IL-23R engagement on effector and memory T cells has been demonstrated to drive IL-17A secretion and Kebir at al. demonstrated that T_H17 cells are able to cross the blood brain barrier and migrate to the CNS more efficiently than T_H1 cells[16;22]. Even though IL-17A itself plays only a minor pathogenic role during CNS-inflammation, it serves as a reliable marker for pathogenicity during EAE[4]. While the polarization of highly purified naïve T cells towards a T_H17 phenotype *in vitro* is IL-23 independent and relies on TGF-β and IL-6 exclusively[11;13;14], the impact of IL-23 on differentiation and expansion of T_H17 cells *in vivo* is a matter of debate[17;18;23]. Cytokine analysis of the CNS-infiltrating cells revealed that wt $CD4^+$ T cells produced robust levels of IL-17A and IFN-g, while CNS-infiltrating pathogenic IL-12Rβ2$^{-/-}$ $CD4^+$ T cells secreted higher levels of IL-17A than wt cells but failed to secrete IFN-g **(Fig. 3A and B)**. This is expected as IFN-g inhibits the polarization towards the T_H17 phenotype and likely acts in an autocrine and short-range paracrine fashion. Interestingly, IL-12Rβ 1-deficient T helper cells, which infiltrate the CNS but do not respond to IL-12 or IL-23, fail to secrete both IL-17A or IFN-γ **(Fig. 3C and D)**. McGeachy at el. just confirmed in a similar experimental design, that IL-23R engagement is vital for T_H17 polarization *in vivo*[21]. T cells lacking the IL-12Rβ2 on the other hand are not impaired in their capacity to invade the CNS, even though they fail to T_H1 polarize. Absolute numbers of polarized T_H1 and T_H17 cells infiltrating the CNS are provided in **Supporting Information Figure 1B.** Our data indicate that *in vivo* IL-23R engagement is critical

for T cells to acquire encephalo-tropism and is essential for the production of IL-17A by CNS-infiltrating T cells.

Loss of IL-12Rb1 alters the ratio regulatory T cells vs. effector T cells in the CNS

TGF-β and IL-6 can polarize T_H17 cells independent of IL-23, TGF-βR engagement enhances the generation of regulatory T cells (Treg). Foxp3 is the master transcription factor of Treg and is expressed by the precursor to both T_H17 and Treg. IL-6 induces the induction of ROR-gt and Runx1 which are thought to be the main switch which re-directs T cell differentiation from the regulatory phenotype towards the effector T_H17 phenotype[24]. To determine whether IL-23 plays any role in Treg or T_H17 lineage commitment during inflammation, we assessed the number of $Foxp3^+$ regulatory $CD4^+$ T cells among the infiltrating leukocytes by cytofluorometrical intracellular cytokine analysis. Even though the total number of effector T cells from the IL-12Rb1$^{-/-}$ population is drastically reduced, within this population, we observed the preferential accumulation of Foxp3 positive cells **(Fig. 3E and F)**. None of the other populations (IL-12Rb2$^{-/-}$ or wt) revealed an elevated percentage of Foxp3 positive cells. Based on this observation, we speculate that IL-23 affects the CNS-tropism and encephalitogenicity of effector T cell, while Tregs are unaffected. It is feasible that without the trophic support by IL-23, effector T cells could undergo apoptosis upon entering the CNS. We did not find any increase in $AnnexinV^+$ cells extracted from the CNS of mice suffering from EAE. In addition, given that we observed a clear expansion and accumulation of IL-12Rβ1$^{-/-}$ T cells in secondary lymphoid organs, we conclude that IL-23 affects CNS tropism, rather than longevity or apoptosis.

Concluding remarks

The role and impact of IL-23 vs. IL-12 on autoimmune T cells remains a subject of intense debate. While *in vitro* generated T_H1 and T_H17 cells can both elicit inflammatory CNS lesions in EAE[25], the factors that control T_H1 and T_H17 polarization, impact on more than just the T cell cytokine profile. T_H17 polarization was demonstrated to be independent of IL-23, but he reports dismissing IL-23 in favour of TGF-β and IL-6 as exclusive T_H17 promoting factors relied on the use of highly purified $CD4^+$ T cells and in vitro expansion[14]. In this report here, we confirm that *in vivo* and during EAE, IL-23 is absolutely critical for T_H17 polarization and the acquisition of encephalitogenicity[21]. In contrast to IL-23, T_H17 signature cytokines are not essential for the development of CNS autoimmunity[4;5]. While IL-17 clearly affects the CNS endothelium, loss of IL-17A & F only leads to reduced inflammation, but not in complete EAE resistance (Haak). Thus IL-23-mediated effects on T cell pathogenicity is a feature far beyond T cell polarization. Here we demonstrate that IL-23 not only promotes T_H17 polarization but also and more critically T cell-

CNS tropism while loss of IL-12 signaling and the polarization towards a T_H1 phenotype is not essential for the tissue infiltrating capacity of auto-reactive T cells.

Materials and Methods

Mice

C57BL/6, IL-12Rb1$^{-/-}$, IL-12Rβ2$^{-/-}$ and RAG1$^{-/-}$ mice were purchased from Jackson laboratories (Bar Harbour, Maine) and bred in house under specific pathogen free conditions. Animal experiments were approved by the Swiss Veterinary Office (13/2006; Zurich, Switzerland) and performed according to federal and institutional guidelines.

To generate BM chimeras, RAG1$^{-/-}$ mice were lethally irradiated with a split-dose of 1100 rad. Donor animals were euthanized with CO_2 and bones (for- and hind legs, hips) were flushed with sterile PBS to obtain BM stem cells. Mutant and wt BM with the respective congenic marker were mixed at a 1:1 ratio. The mixture was verified by flow cytometry with anti-CD45.1 (A20) and anti-CD45.2 (104) mAb (BD Pharmingen). In total, 2x10^7 cells were injected i.v. per mouse and to prevent bacterial infection 0.2% BORGAL was added for two weeks to the drinking water.

Induction of EAE

6-8 weeks after reconstitution BM-chimeras were immunized subcutaneously with 200 µg of $MOG_{35–55}$-peptide emulsified in CFA into the flank. Clinical disease was scored daily as follows; 0, no clinical disease, 1 - limp tail, 2- impaired righting reflex, 3 - hind limb paralysis, 4 - moribund, 5 - dead.

Fluorocytometric analysis of splenocytes and mononuclear cells from the CNS

Extraction of mononucleated cells from inflamed CNS tissue was performed as described previously[26]. Mononucleated cells were stimulated in RPMI1640 containing 10% FBS (both from Invitrogen) supplemented with PMA (50ng/ml) Ionomycin (500ng/ml) and BD GolgiPlug™ (1µl/ml) for 5h at 37ºC. Fluorocytometric analysis of surface marker expression was performed as described[26]. Intracellular cytokine staining was performed with the Cytofix/Cytoperm™ Plus Kit (BD Bioscience) according to manufacturer's directions. Antibodies used: anti-IL-17A (TC11-18H10), anti-IFN-γ (XMG1.2) (BD Pharmingen), anti-Foxp3 (FJK-16s) (eBioscience).

Statistics

The significance of the difference in percentages/ratios of different cell populations was evaluated using a two-tailed Student's *t* test. *P* values of less than 0.05 were considered significant.

Acknowledgements:

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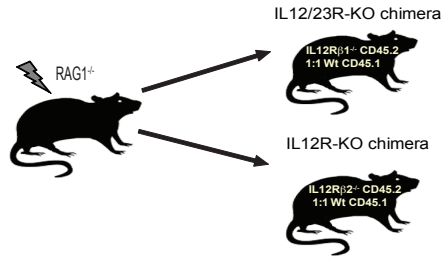
Figure 1. Generation of mixed BM chimeras and EAE development in these animals
(A) Generation of the mixed BM chimeras. Lethally irradiated RAG1$^{-/-}$ mice were reconstituted with either equal numbers of wt and IL-12Rb1$^{-/-}$ BM cells (IL-12/23R KO chimera) or with equal numbers of wt and IL-12Rb2$^{-/-}$ BM cells (IL-12R KO chimera). BM-donors are congenically different (CD45.1/2) and thus traceable. **(B)** Clinical scores after MOG_{35-55}-induced EAE (no significant difference between the two different groups). Error bars represent ± SD, data are representative of three independent experiments. **(C)** Detailed clinical development of EAE in IL-12/23R KO and IL-12R KO BM chimeras.

Figure 2. Absence of IL-12Rb1 engagement but not IL-12 signaling results in the inability of T cells to invade the CNS leading to their accumulation in peripherial lymphoid organs. IL-12R KO and IL-12/23R KO chimeras were immunized with MOG_{35-55}/CFA. At peak disease (16dpi), CNS-infiltrating leukocytes, cells from peripheral lymphoid organs were isolated from either IL-12/23R KO chimeras or IL-12R KO chimeras and stained for congenic markers. The distribution of different cell populations was assessed by flow cytometry. Plots shown are gated on CD4$^+$ CNS-derived T cells. **(A, B)** CD4$^+$ T cell compartment (CNS-infiltrating leukocytes) in the IL-12R KO or IL-12/23R KO chimeras, respectively. **(C, D)** CD4$^+$ T cell compartment (spleen and lymph node) in the IL-12R KO or IL-12/23R KO chimeras, respectively. The panels to the right display individual percentages of all experiments combined. Data are representative of at least four independent experiments. Two-tailed Student's *t* test was used to calculate p values.

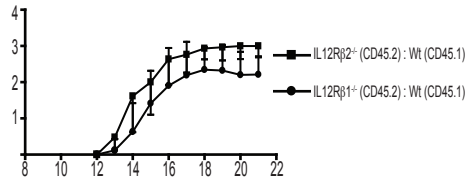
Figure 3. Loss of IL-12Rb1 engagement leads to decreased production of proinflammatory cytokines by CD4$^+$ T cells and preferential accumulation of Foxp3$^+$ CD4$^+$ T cells in the inflamed CNS. Expression of IL-17A and IFN-g was measured by intracellular cytokine staining. Dot plots are gated on CD4$^+$ CNS-derived T cells. Percentages of IL-17A$^+$ and IFN-g$^+$ cells are given in the quadrant corners. **(A, B)** Cytokine production by CNS-infiltrating CD4$^+$ T cells derived from the IL-12R KO BM chimeras, IL-17A and IFN-g, respectively. **(C, D)** IL-17A and IFN-g secretion by CNS-infiltrating CD4$^+$ T cells from IL-12/23R KO BM chimeras. The expression of Foxp3 was measured by intracellular cytokine staining. Dot plots are gated on CD4$^+$ CNS-derived T cells. **(E)** Foxp3 expression in the IL-12/23R KO BM chimeras. **(F)** Foxp3 expression in the IL-12R KO BM chimeras. Data are representative of at least 3 independent experiments. The panels to the right display individual percentages of all experiments combined. 2-tailed Student's *t* test was used to calculate p values.

Figure 1

A



B



C

	Day of onset	Incidence	Max. score
IL-12Rβ1-/- : wild type	13 ± 0.5	95 % (22 / 23)	2.5 ± 0.6
IL-12Rβ2-/- : wild type	12 ± 0.3	100 % (25 / 25)	2.92 ± 0.29

Figure 2

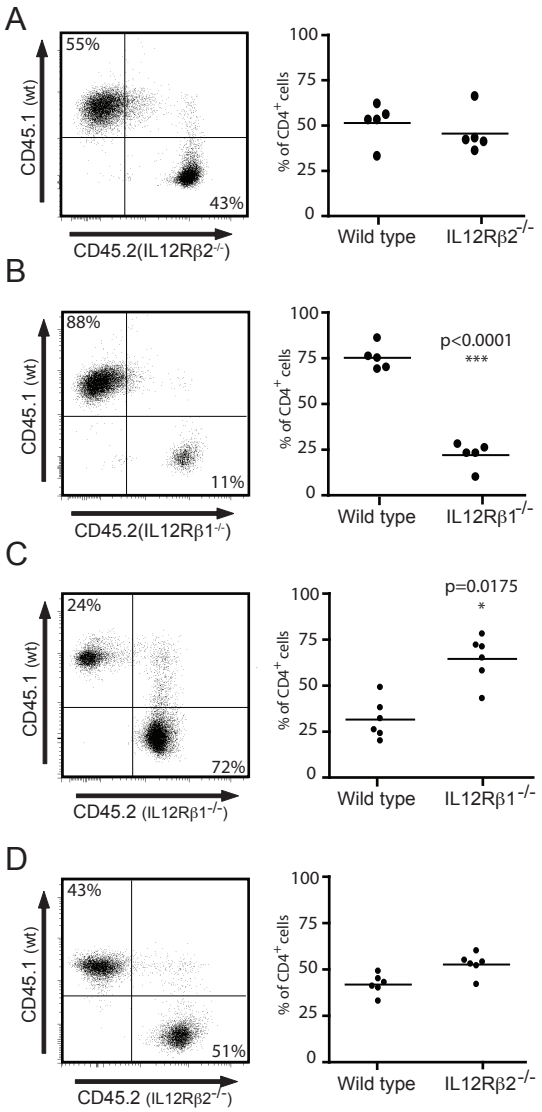
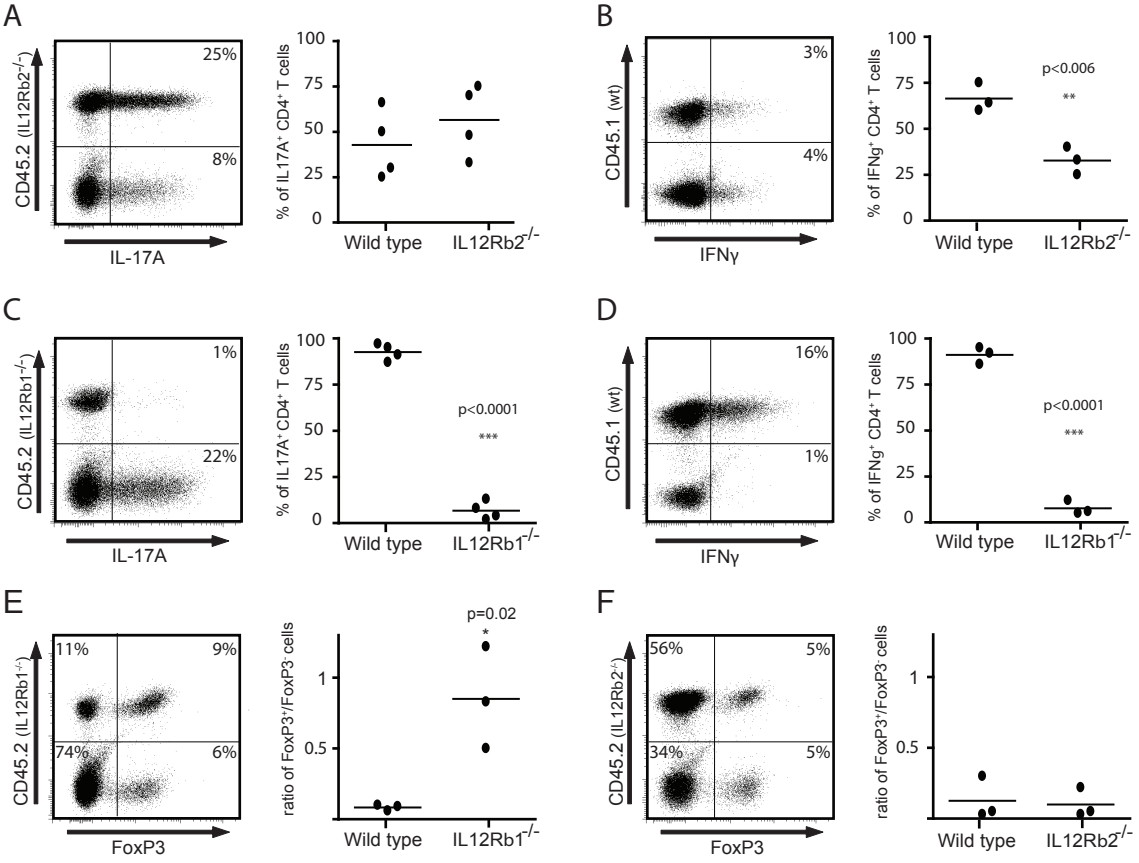
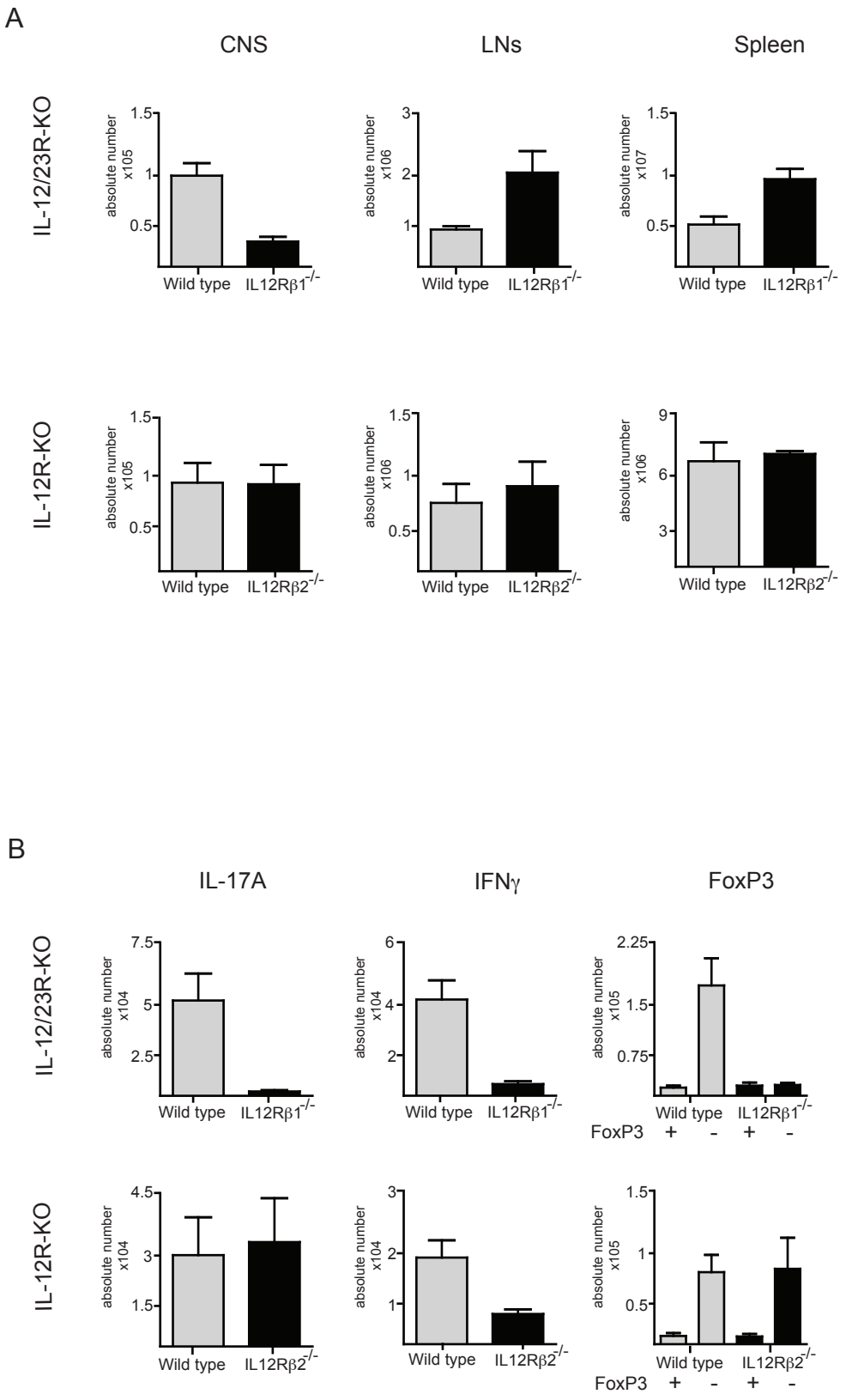

A
55%
43%
CD45.1 (wt)
CD45.2(IL12Rβ2-/-)
% of CD4+ cells
100
75
50
25
0
Wild type
IL12Rβ2-/-
B
88%
11%
CD45.1 (wt)
CD45.2(IL12Rβ1-/-)
p<0.0001
***
% of CD4+ cells
Wild type
IL12Rβ1-/-
C
24%
72%
CD45.1 (wt)
CD45.2 (IL12Rβ1-/-)
p=0.0175
*
% of CD4+ cells
Wild type
IL12Rβ1-/-
D
43%
51%
CD45.1 (wt)
CD45.2 (IL12Rβ2-/-)
% of CD4+ cells
Wild type
IL12Rβ2-/-

Figure 3


A
25%
8%
CD45.2 (IL12Rb2-/-)
IL-17A
% of IL17A+ CD4+ T cells
Wild type
IL12Rb2-/-
B
3%
4%
CD45.1 (wt)
IFNγ
p<0.006
**
% of IFNg+ CD4+ T cells
C
1%
22%
CD45.2 (IL12Rb1-/-)
p<0.0001
***
D
16%
1%
E
11%
9%
74%
6%
FoxP3
p=0.02
*
ratio of FoxP3+/FoxP3- cells
F
56%
5%
34%
5%

Supporting Information Figure 1



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Generation of mice with conditionally targeted *il23r* locus

LoxP mediated conditional gene targeting was used since the conventional gene targeting would have not been useful in our case because of the following reasons:

- The conventional gene targeted animal already exists and shows the same EAE resistance phenotype as IL-12Rβ1$^{-/-}$
- Does not allow us to specifically reveal the impact of IL-23 on different cells types of the immune system

The conditional allele of *il23r* was planned in a way to flank exon 6 by loxP sites. The gene structure of *il23r* was analyzed by computational analysis and the analysis reveled that removing exon 6 causes frame shift mutation and ultimately leads to the deletion of an important domain (WSxWS), which is responsible for ligand binding.

Targeting strategy

Introducing the flanking loxP sites and the selection markers into the mouse genome was achieved by replacing the wild type exon 6 and its surrounding region with a targeting construct pRapidflirtIL23r#17 by means of homologous recombination. The targeting vector consists of resistance genes that allow the proper identification of homologous recombination in ES cells, two homologous arms, which enable the cross over event in mouse ES cells, the wild type exon flanked by loxP sites which allows the subsequent removal of the exon by Cre recombinase and two frt sites in order to remove the Neo cassette from the genome. The loxP sites flanking exon 6 are placed into intronic regions thereby not interfering with proper splicing and the animal derived from the targeted ES cell behaves as wild type.

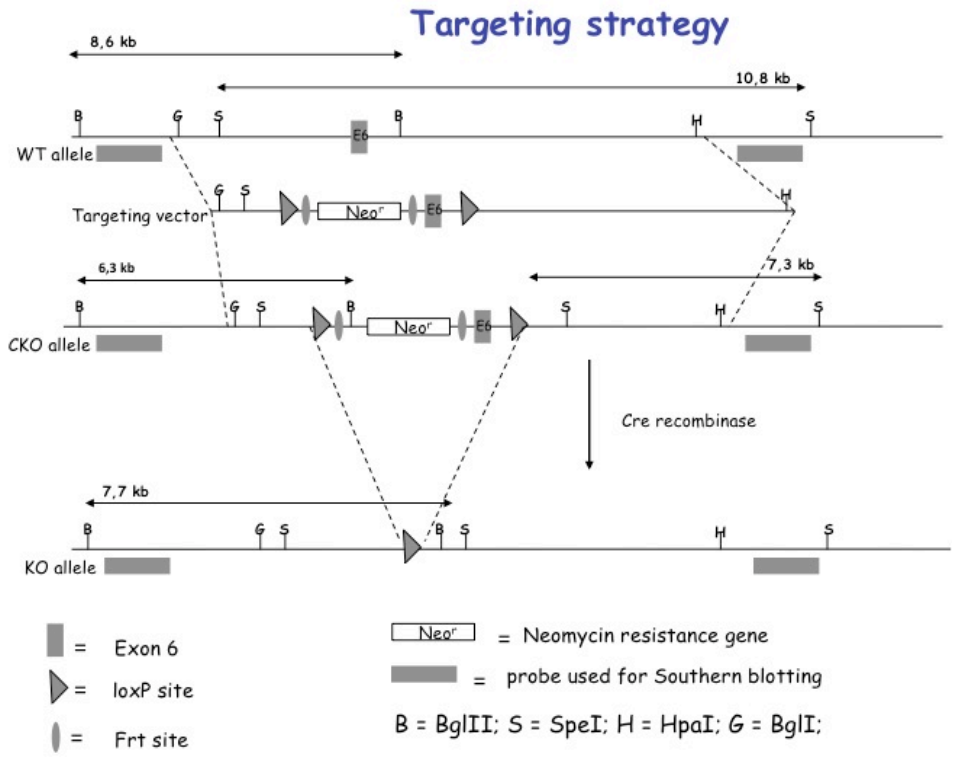


Figure 4. The targeting strategy of the generation of the IL-23R conditionally targeted mouse
The figure shows the most important DNA elements used for the generation of the targeted il23r allele. It indicates the external Southern blot probes used for the screening.

Description of the targeting vector

The original vector, pRapidflirt contains an Amp resistance gene, which makes it possible to amplify it in bacteria. It contains a positive and a negative selection marker. This setup is commonly used for reducing the ES cell clones with random integration events. The final targeting vector was achieved by conventional cloning.

As a negative selection marker, herpes simplex virus type 1 thymidine kinase (HSV-tk) is used. To select for the absence of HSV-tk expression, gancyclovir was used. This drug will kill cells expressing the HSV-tk while it does not harm cells lacking the product of gene indicating proper homologous recombination. Typically, from a 2-fold to 20-fold enrichment of properly targeted clones can be achieved by the administration of gancyclovir. This procedure is not 100% effective, since some random integration ES cell clones can escape the negative selection procedure. This survival of cells can be explained by loss of function of the thymidine kinase due to methylation or mutation. The thymidine kinase-ORF is placed on the 3'-end of the targeting

construct and if proper homologous recombination happens it does not get integrated into the genome.

The positive selection marker is the neomycin gene, which metabolizes G418, which is a toxic compound for eukaryotic cells. During this process, in contrast to the HSV-tk, the presence of the Neo resistance gene is vital for ES cell clone survival. The Neo^r ORF is flanked by FRT sites, which later on allow the specific excision of the unnecessary resistance gene from the mouse genome by the use of Flippase recombinase.

During the generation of the targeting vector, three different wild-type genome segments were amplified and subsequently cloned into the pRapidlirt plasmid. It has been shown that frequency of homologous recombination increases with the length of the homology between the targeting vector and target locus. The left homology arm was 5kb long while the right arm was 7 kb in length. The resulting ready-to-go plasmid was 19 kb.

PCR amplification of homology arms and exon 6

In order to generate the targeting vector, the homologous arms and the wild type exon were amplified by HiFi polymerase. The PCR products after purification were subcloned into pGEM-T easy vector and later on, their integrity was confirmed by restriction digest and DNA sequencing. The homologous arms were amplified from bacterial artificial chromosomes (BAC), which contained the genomic region in the proximity of the *il23r*.

Primers used for the amplification of the homologous arms contained sequences of restriction enzymes, which later on made it possible to proper cloning into the final targeting vector.

The exon 6 was amplified from the BAC and contained the ORF of exon 6 and the splicing acceptor site. The fragment was amplified by the use of primers exon6 fw and exon 6 rev. The expected length of the fragment is 916 bp and the amplified DNA piece is shown on Fig. 5.

The right and left homologous arms were amplified from the same BAC. right arm fw and right arm rev primers were used for PCR amplification and the expected fragment size was 5.6 kb for the right arm and the 5' end contained an artificially integrated restriction site, SpeI, which later on helped to identify proper homologous integrant. The left arm was 4 kb long and for its amplification left arm fw and left arm rev primers were used.

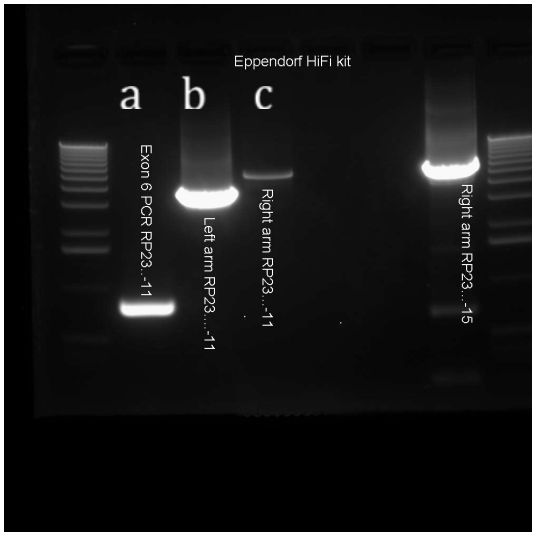


Figure 5. Agarose gel showing the PCR amplified homologous arms and exon 6
The homologous arms and exon 6 were amplified by PCR and after purification were cloned into pRapidflirt, subsequently yielding the targeting construct.
a.) exon 6 b.) left homologous arm c.) right homologous arm

Generation of the targeting vector

Exon 6 and the surrounding region was cut out from the pGEM-T easy vector and was subcloned into the targeting pRapidflirt plasmid by the use of SalI restriction enzyme. After successful integration of the fragment, an 8kb long plasmid was generated.

As it described previously, the left and right homologous arms were cloned into pGEM-T Easy vector. From the pGEM-T Easy plasmid they were cut out with NotI/ClaI and XhoI/FseI, respectively. After gel purification, the fragments were cloned into the pRapidflirt, which already contained exon 6. In the first step, the right arm was placed into the targeting plasmid resulting a 13.5 kb long construct. In the last cloning step, by cloning the purified, left homologous arm into the pRapidflirt, the final targeting vector was generated. This construct had a length of 19 kb.

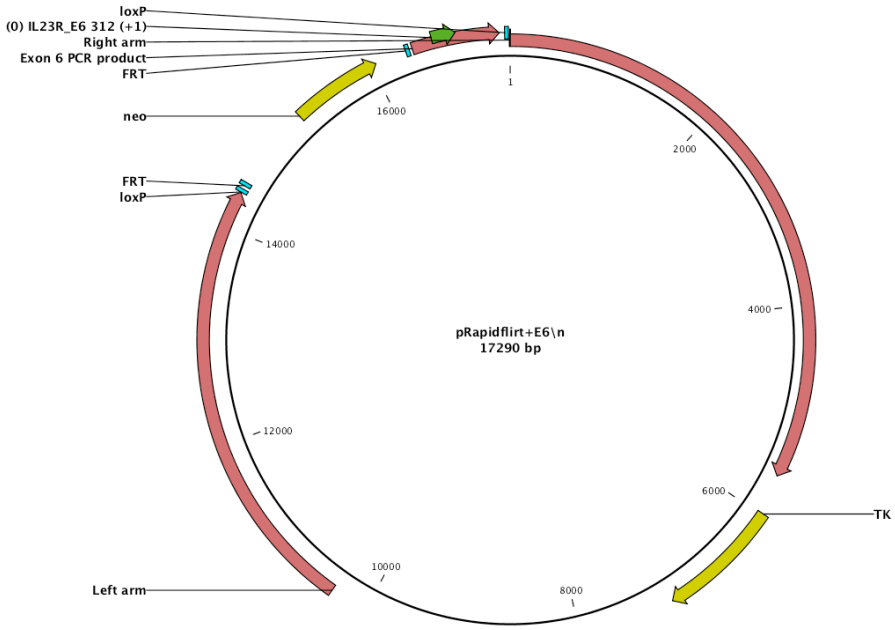


Figure 6. Final targeting construct

In silico representation of the targeting vector after successful cloning of the homologous arms and exon 6 into pRapidflirt. The final vector was sequenced and analyzed by restriction digest. Subsequently this construct was used for ES cell transfection.

Verification of the targeting vector prior ES cell transfection

In order to verify the integrity of the targeting plasmid, several restriction digests were performed. Later on, the important sequences that play an important role in the selection process or later on in the removal of exon 6 were sequenced. The DNA sequencing covered the regions containing the loxP and FRT sites, Neo^r ORF and exon 6.

The restriction digest showed that no major DNA sequence changes happened during cloning and the digest did not reveal unexpected digestion pattern. The sequencing proved that the loxP and FRT sites together with exon 6 were mutation free.

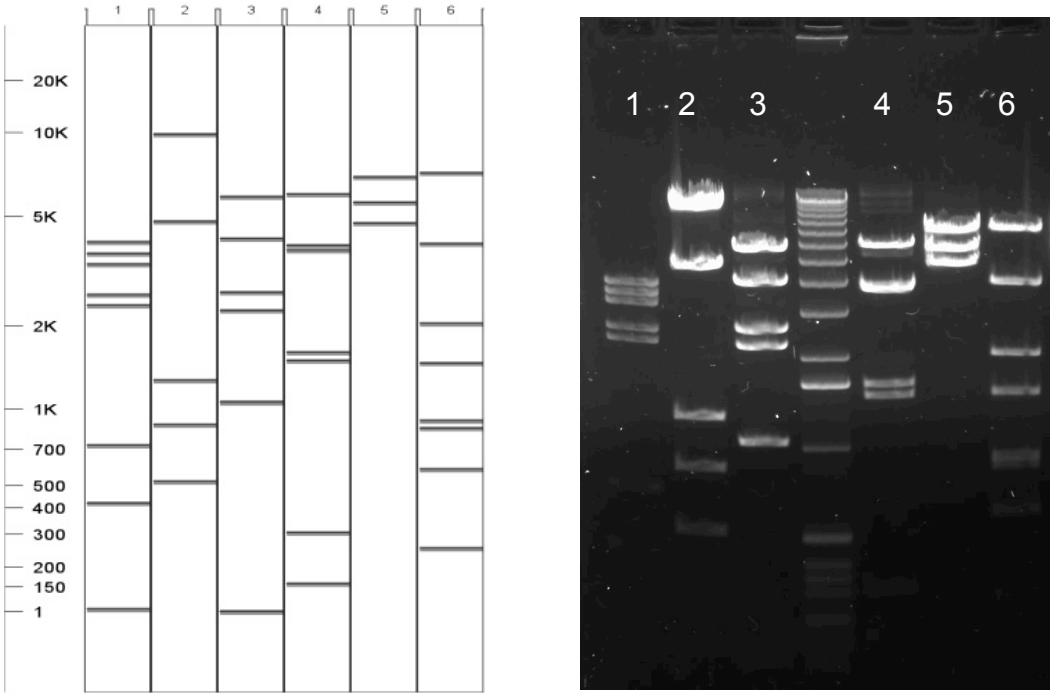


Figure 7. Restriction digest of the targeting vector

The purified vector was cut with different enzymes in order to verify its integrity. The digestion pattern correlated with the in silico generated data.

1. EcoRI, EcoRV *2. HindIII* *3. SacI* *4. XbaI*

5. ClaI XhoI FseI *6. SalI NcoI NotI*

Generation of ES cells containing the targeting vector

The procedure from transfection of ES cells until morula aggregation includes several steps. In the first step, ES cells were transfected with the properly linearized targeting construct pRapidflirtIL23r#17, and after transfection ES cells underwent double-selection procedure. G418 was added to media in the positive selection step in order to enrich those ES cell clones, which harbored the targeting vector. Subsequently, surviving colonies were exposed to ganciclovir in order to exclude non-homologous recombinant. ES cell colonies after the double-selection procedure were picked and were screened for proper homologous recombination by Southern blot.

Transfection of murine ES cells

We performed several transfection experiments using electroporation on different embryonic cell lines. Our first several attempts were unsuccessful by using either Bruce4 C57Bl/6-derived ES cell lines or B6/129 F1 ES cells. Both cells require the presence of feeders on the surface of the culture plate. Unfortunately, for an unknown reason our feeder cells always died and this fact always deteriorated significantly the survival rate of the ES cells. After these failed attempts, to circumvent the feeder cell problem we decided to use federless JM8/B6 ES cells. We performed two rounds of electroporation with the linearized targeting vector, pRapidflirtIL23r#17. Linearization was performed with ClaI restriction enzyme digest of the targeting vector. (Fig. 8.)

Figure 8. Linearization of the targeting vector
Prior to electroporation into ES cells, the targeting vector was linearized by ClaI to facilitate homologous recombination. Number 2 shows the linearized, cut vector and number 3 is the super coiled vector. Number 1 is the further digested (ClaI, SpeI), linearized vector. The additional bands show the integrity of the vector. Arrow indicates the 3 kb band.

The transfected ES cells were plated onto collagen-coated plates and after a 24h recovery period were grown under selective conditions. We plated out 10^6 transfected ES cells onto a 10-cm plate. As the G418 containing selection medium was added to the cells, many of them died due to lack of integrated targeting vector. Ganciclovir treatment was used to enrich homologous recombinant after positive selection and this resulted in further enrichment of positive ES cell colonies. The HSV-tk ORF cannot be integrated into the genome if homologous recombination happens but it is present when non-homologous recombination event occurs thereby causing cell death.

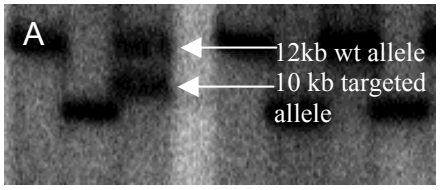
Identification of positive ES cell clones

Targeted insertion of the pRapidflirtIL23r#17 targeting construct resulted in homologous recombination into the *il23r* locus in murine ES cells. In order to identify homologous recombinant the surviving and individually expanded ES cell clones were screened by Southern blot. During Southern blot pre-designed, radioactively labeled 5' and 3' DNA probes allow the identification of homologous recombinants in ES cells. Our 5' prime probe on StuI digested wild-type gDNA gave a signal at the height of 12kb, whereas the properly targeted allele showed a wild-type signal with the previously mentioned 12 kb signal and a 10 kb band, indicating the targeted allele. For the 3' probe, based on *in silico* calculations, a 12 kb long wild type band and a 7,3 kb long targeted allele band were expected. During the generation of the targeting construct, in the proximity of the 3' loxP site, a restriction site was introduced by PCR in order to detect the proper integration of both loxP sites. If the homologous recombination happens between the 5' arm and the exon 6 and their gDNA homologous, the Southern blot analysis would reveal a 10 kb long fragment instead of the proper 7,3 kb band.

The 5' and 3' Southern blot probes were tested on gDNA prior to the screening process. gDNA from different sources was used with the combination of different restriction enzymes. The expected band size was 12 kb and after testing with several enzymes we chose StuI and SpeI as these two enzymes gave the most reliable results.

Screening of the transfected JM8 ES cell clones

All together, 550 ES cell clones were screened for homologous recombination with the 5' probe by Southern blot hybridization. In the first round, those clones were selected for further evaluation, which showed with the 5' probe a 12 and a 10 kb long double band. These ES cell clones most likely harbored the targeting construct at the right location. To verify the identity of these clones, we performed Southern blot using the 3' probe. The following pictures show the positive clones identified by southern blot.



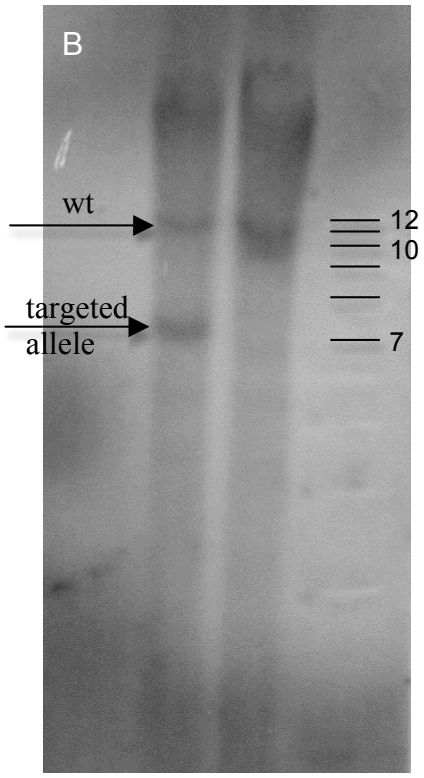


Figure 9. 5' and 3' Southern blot screening results

5.A. shows a double band as a result of proper homologous integration. The upper, 12kb band corresponds to the wild type allele while the lower 10kb fragment shows the integration of the targeting vector. 5. B. shows the result of the 3' probe Southern blot. The upper 12kb long fragment is the wild type allele while the other 7.3 kb lower band indicates the targeted allele.

The 5' probe Southern blot analysis revealed 12 clones with integrated targeting vector. The 3' analysis reduced this number to only two properly targeted clones. Unfortunately, in the other ten cases, for an unknown reason the homologous recombination instead of occurring between the 5' and 3' homology arm, the crossovers happened linking the 5' homology arm with its gDNA complementary sequence and the exon 6 with its homologous sequence in the mouse genome. By the use of a specific analysis program (RepeatMasker) we revealed that the 3' prime homology arm contains a high percentage of interspersed repeats which might inhibit proper homologous recombination and the recombination machinery favored the shorter but less repetitive sequence for crossover formation.

After transfection of ES cells with our pRapidflirtIL23r#17 construct 550 ES cell clones were picked and screened. Out of these clones, only two proper homologous recombinants were identified by Southern blot. This results in a relative recombination frequency of 1 in 275. The positively identified clones were expanded and stored at -80 degrees prior to further manipulations.

PCR verification of the positive ES cell clones

We established a PCR method in order to validate the identity of the positive clones, which were identified previously by Southern blot. As it is discussed earlier, the presence of the 3' loxP site is always hard to be detected. The PCR enabled us to localize the position of the integrated construct and by digesting (SpeI) the resulting amplicon, even the presence of the 3' loxP site can be proven. The primers are designed in a way that the PCR on wild type DNA does not yield a product while the amplification of the properly targeted allele shows a 8.2 kb long fragment.

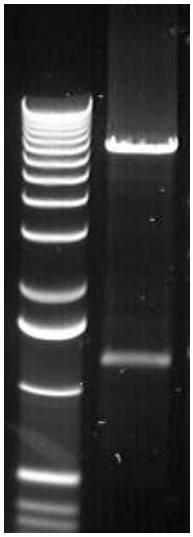


Figure 10. Gel picture of the digested PCR product
After amplifying ES cell gDNA containing the region where the 3' loxP site is localized, the amplicon was further digested with SpeI and the digest yielded 1.4kb and 6.8 kb fragments, indicating the presence of the 3' loxP site.

Discussion

Current view on autoimmune diseases in the CNS

Multiple sclerosis and experimental autoimmune encephalomyelitis, the animal model of MS, are autoimmune demyelinating diseases of the CNS. Shortly after the discovery of the T_H1 subset, it was already proposed that CNS tissue injury in organ-specific autoimmunity was initiated by IL-12 stimulated, IFN-γ-producing myelin-specific T cells (Leonard et al., 1996). This hypothesis was reinforced by the observation that either the deletion of the IL-12p40 or the IL-12Rβ1 subunit leads to complete EAE resistance after active immunization with the immunodominant peptide, MOG_{35-55}. (Segal et al., 1998; Zhang et al., 2003b)

In contrast to these observations, subsequent studies with mice deficient in either IL-12p35/IL-12Rβ2 (unique components of IL-12 or IL-12 receptor, respectively) or the T_H1 hallmark cytokine IFN-γ remained susceptible to EAE induction and even more they showed elevated disease severity questioning the exclusive role of IL-12 in the initiation and maintenance of EAE (Becher et al., 2002a; Ferber et al., 1996; Zhang et al., 2003a).

The paradox was solved by the discovery of IL-23, which is a cytokine composed of a common IL12p40 subunit and a unique p19 subunit (Oppmann et al., 2000). It is produced mostly by activated antigen presenting cells such and dendritic cells and macrophages. Its receptor is expressed on the surface of activated T cells, NK cells and APCs and it is a heterodimer of IL-12Rβ1 (shared with IL-12) and IL-23R (unique subunit) (Parham et al., 2002).

In the past 7 years numerous studies demonstrated that either the genetic deficiency or *in vivo* blockade by antibodies of IL-23p19 subunit results in complete EAE resistance (Cua et al., 2003b). Furthermore, several studies indicated that IL-23 treatment enhances the pathogenic potential of MOG_{35-55} reactive T cells. In line with these observations, it became apparent that IL-23 rather than IL-12p70 is critical for the development of autoimmune inflammation of the CNS.

Since the initial discovery of IL-23, it only became obvious that the lack of IL-23 leads to defects in the generation of the inflammatory T_H17 cells. The initial assumption was that IL-23 is required for the generation, expansion, or survival of IL-17-producing cells. Lately, its pivotal role in the generation of T_H17 cells has been questioned by several studies which showed that the lack of IL-17A alone or in combination with IL-17F does no lead to complete EAE resistance as it is observed in IL-23-deficient animals. Despite many years of intense research, the mechanism underlying the disease promoting effects of IL-23 still remains elusive and no single T helper

subset or cytokine has been described to be mandatory for the development of autoimmune inflammation in the CNS (Gutcher and Becher, 2007; Haak et al., 2009a; Kreymborg et al., 2007).

The impact of IL-23 on T cell polarization under inflammatory conditions *in vivo*

In order to study the function of a certain cytokine, several methods can be applied but among them the most elegant way of studying the function of a molecule is by generating a mouse genetically deficient in that molecule. IL-23-deficient animals were created but their EAE resistance made it actually impossible to study the function of this pro-inflammatory cytokine in the context of autoimmunity. In order to circumvent this difficulty, we generated mixed BM-chimeric mice, which enabled us to reveal the IL-23-dependent T cell behavior during EAE. By creating an environment in which half of the leukocytes had a wt genotype, while the other half was unable to respond to IL-23 we could investigate the effect of IL-23 on T cells under inflammatory conditions. The use of bone marrow stem cells derived either from the common (IL-12Rβ1$^{-/-}$), or from the specific (IL-12Rβ2$^{-/-}$) IL-12 receptor subunit deficient animals in the generation of chimeric animals allowed the assessment of the behavior of wild-type and receptor deficient cells in the very same animal under inflammatory conditions.

The lack of either IL-12 or IL-12/23 responsiveness did not impair the capacity of stem cells to repopulate the immune compartment and by the use of congenic markers (CD45.1 wt; CD45.2 cytokine receptor deficient cells) we could clearly distinguish between the two cell populations and evaluate their distribution within the systemic immune compartment.

EAE developed in both groups with comparable severity levels although in animals where IL-12-unresponsive cells constituted half of the immune system a slightly exacerbated disease progression was observed. This could be due to the loss of the inhibitory effect of IL-12 on the development of the pathogenic T cell population. It was already well described that polarizing factors and hallmark cytokines of the T_H1 subset ameliorates EAE by suppressing the activity/differentiation of pathogenic T cells (Becher et al., 2002a; Gran et al., 2004a; Gran et al., 2002).

T cells unable to respond to IL-12 were present in the peripheral lymphoid organs in the same number as wild type T cells while in contrast, cells lacking the ability to engage IL-23 accumulated in the spleen and lymph nodes. It seemed that their proliferation was not affected by the absence of IL-23 but it appeared that after priming they failed to leave the lymphoid compartment and migrate to their target organ.

We observed a massive infiltration of mononuclear cells into the brain and spinal cord in both groups. IL-12R-deficient T cells invaded the CNS as efficiently as their wild type counterparts and the lack of IL-12 signaling exerted no impact on the migratory capacity of T cells. In contrast, T cells being unable to respond to IL-23 stimulation failed to invade the CNS while wild type cells were overrepresented in the inflamed organ.

So far, IL-23 has not been associated with migration of pathogenic cell populations into their target organs. In previous publications only its impact on the generation on T_H17 cells has been emphasized. Here, we provided the first evidence that the loss of IL-23 signaling negatively influences the encephalo-tropism of T cells, whereas the lack of IL-12 receptor engagement does not inhibit the capacity of T cells to infiltrate the CNS during EAE. McGeachy and her colleagues by using the very same model of mixed bone marrow chimeric mice observed an identical phenotype thereby confirming our finding (McGeachy et al., 2009a).

Deletion of the major signal transducer molecule of IL-23 signaling results in a similar phenotype. $CD4^+$ T cells bearing mutation in the STAT3 gene fail to acquire pro-inflammatory properties and to traffic into the CNS tissue (Liu et al., 2008b). This finding suggests that both the deletion of IL-23 receptor or members of its signaling cascade negatively influences the homing capacity of encephalitogenic T cells and most likely in the absence of IL-23 signaling T cells can not complete their differentiation program towards the T_H17 direction.

The absence of IL-12Rβ1 deficient T cells in the CNS could be due to altered chemokine receptor expression pattern. It has been observed that on the surface of T helper cells CCR5 and CXCR3 are important receptors for the migration into inflamed sites and it is likely that IL-23 mediates the expression of these regulatory molecules on T cells. Pathogenic T cell populations migrate poorly into the inflamed organ and they rather traffic to the spleen when CCR5/CCR6 and/or CXCR3 are not present on the cell surface (Acosta-Rodriguez et al., 2007; Aranami and Yamamura, 2008; Izikson et al., 2000; Lametschwandtner et al., 2004; Tran et al., 2000; Yamazaki et al., 2008).

The lack of IL-23 responsiveness inhibits the production of pro-inflammatory cytokines in the CNS by MOG-reactive T cells

We analyzed the cytokine expression pattern of CNS infiltrating cells. Even though, IL-17 has been shown to play only a minor role during CNS inflammation, it still serves as a reliable pathogenicity marker for infiltrating autoreactive T cells (Haak et al., 2009a). Cytokine analysis of infiltrating $CD4^+$ T cells revealed that wild type T cells produce robust quantities of IFN-γ and IL-17A in the CNS whereas T cells unresponsive to IL-12 produced higher amounts of IL-17 and

failed to secrete IFN-γ. This observation is not surprising, hence the fact that IL-12 negatively regulates IL-17 expression is well described and IL-12 is the main inducer of INF-γ production under inflammatory conditions (Liu et al., 2008a; Mathur et al., 2006; Rangachari et al., 2006). Surprisingly, T cells lacking the IL-12Rβ1 subunit showed a drastically decreased production of IL-17A when we compared to wild type cells. It was already described that IL-23 stimulation is required to induce IL-17 secretion by memory $CD4^+$ T cells *in vitro* but the same effect has not yet been demonstrated *in vivo* under inflammatory conditions. IFN-γ production was completely abolished in the absence of IL-23 signaling as well, which indicated the role of IL-23 beyond its influence on the production of pro-inflammatory cytokines. It is likely that IL-23 plays major role in the maturation process of T cells and in the absence of IL-23, T cells remain in a naïve stage and these immature T cells are unable to leave the peripheral lymphoid organs although their proliferation is not impaired.

Our speculation is further supported by the fact that in the inflamed CNS we observed an increased frequency of FoxP3-expressing regulatory cells among the IL-12Rβ1-deficient T cells when we compared to either the wild type or the IL-12Rβ2 deficient population. However, this preferential accumulation and differentiation of T cells towards the regulatory phenotype is not entirely unexpected. It has been already shown that T_H17 cells next to IL-17 production secret the T_H1 cytokine IFN-γ already questioning the stability of the T_H17 lineage (Lee et al., 2009). It was described that FoxP3 interacts with Rorγt, the main transcription factor of T_H17 differentiation, and the balance between these two transcription factors decides about the fate of activated, antigen specific T cells (Ichiyama et al., 2008; Zhou et al., 2008). It is likely that in the absence of IL-23, the activity of Rorγt is suppressed by FoxP3 and T cells instead of acquiring pro-inflammatory properties, they rather differentiate towards regulatory phenotype (Awasthi et al., 2008; Locksley, 2009; Zhou et al., 2009b).

Lack of IL-23 engagement inhibits GM-CSF secretion by invading MOG-specific T cells

In mixed bone marrow chimeras we already observed that the absence of IL-23 stimulation highly influences the behavior/migration of T cells during EAE development. T cells unable to engage IL-23, they accumulated in the peripheral lymphoid organs and failed to invade the CNS. This finding indicated the crucial role of IL-23 in directing MOG primed T cells into their target organ. Furthermore, IL-12Rβ1$^{-/-}$ T cells that accumulated in the CNS were unable to produce pro-inflammatory cytokines such as IL-17A and IFN-γ.

By further analyzing cytokine secretion of T cells in the CNS, we could detect that CNS invading T cells produced another pro-inflammatory molecule, GM-CSF. Although GM-CSF is thought to play important role in the differentiation of myeloid cells, recently it has been demonstrated that it has pro-inflammatory properties and its presence contributes to EAE development. The interesting observation was that T cells lacking only the IL-12Rβ2 subunit (unresponsive to IL-12 alone) produce elevated levels of GM-CSF compared to wild type cells. Earlier studies already showed that the lack of IL-12 leads to more severe EAE development. The EAE hyper-susceptible phenotype in these animals was claimed to be due to increased IL-17 secretion in the absence of T_H1 cytokines and was not connected to higher GM-CSF levels (Rangachari et al., 2006; Zhang et al., 2003a).

In contrast to wild type or IL-12Rβ2 deficient cells, IL-12Rβ1 mutant cells failed to produce GM-CSF in the brain. This affect cannot be attributed to the loss of IL-12 signaling in T cells, most likely it was the result of IL-23 unresponsiveness. It was already described that IL-23 *in vitro* up-regulates the expression of GM-CSF and GM-CSF is essential for EAE development although so far no causative link has been proposed between IL-23 and GM-CSF *in vivo*. Until this day, GM-CSF is the only known cytokine, whose production is directed by IL-23 in T cells and its presence is vital for EAE development.

T_H1 associated factors are negative regulators of GM-CSF production

After revealing that T cells lacking responsiveness to IL-23 failed to produce GM-CSF but in contrast missing IL-12 signaling enhanced the secretion of this pro-inflammatory cytokine we wanted to generate GM-CSF-producing polarized T_H cells *in vitro*.

It has been shown that IL-17-producing cells can be generated *in vitro* although their *in vivo* relevance still remains a matter of debate. TGF-beta is needed, together with IL-6, for the induction of IL-17 by naïve T cells and blockage of T_H1 and T_H2 hallmark cytokines enhances the effect of these two factors on IL-17 production (Bettelli et al., 2006; Mangan et al., 2006; Weaver et al., 2007). Interestingly, addition of TGF-beta, IL-6 and αIFN-γ did not induce GM-CSF expression by T helper cells but blockade of IL-12 and IFN-γ greatly enhanced GM-CSF production.

In order to verify the notion that IFN-γ and IL-12 behave as negative regulators of GM-CSF secretion *in vivo* we isolated splenocytes from either IFN-$\gamma^{-/-}$ or IL-12Rβ2$^{-/-}$ animals. The absence either of IL-12-responsiveness or of IFN-γ production enhances the expression of GM-CSF by T

cells. Both cytokine deficient animals develop more severe EAE than wild type mice, suggesting their regulatory role in EAE. Previously this effect has been attributed to elevated IL-17 production as a direct result of higher numbers of IL-17-producing cells during EAE in the CNS. So far, nobody has analyzed GM-CSF production in these animals under inflammatory conditions in the CNS and there has been no link ever made between elevated GM-CSF production and exacerbated disease development in IFN-$\gamma^{-/-}$ or IL-12Rβ2$^{-/-}$ animals. IL-12 and IFN-γ play a crucial role in the effector function of T_H1 cells and it seems probable that by being vital for enhancement of T_H1 development (Trinchieri, 2003), they play an inhibitory role in the generation of pathogenic T cells expressing GM-CSF.

The role of GM-CSF during adoptive transfer

After establishing culture conditions favoring GM-CSF production by T cells, we analyzed the *in vivo* function of *in vitro* generated GM-CSF-producing T cells. In the last several years many studies showed that different T cell subsets differentiated under varying *in vitro* conditions can play a role in the induction of EAE but very often with contradictory results. Unfortunately, the comparison of results derived from different studies is difficult because seldom they use the same culture conditions or T cell population.

It has been observed that IL-17-producing T cells infiltrate into the CNS prior to the development of clinical symptoms of EAE and they induce the production of molecules (matrix metaloproteinase), which permeabilize the blood brain barrier thereby facilitating the recruitment of inflammatory cell into the CNS. In contrast, significant infiltration of T_H1 cells can be only observed after disease onset. Other groups found that highly purified T_H1 and T_H17 cell populations separately cannot induce disease and the interplay of the two subsets is needed for CNS tissue invasion. It was even shown that T_H17 cells although do not require the presence of IL-23 for their differentiation *in vitro* but it is vital for acquiring encephalitogenic properties (McGeachy et al., 2007). Interestingly, transfer of MOG-specific T cells containing different proportions of T_H1 and T_H17 cells direct the localization of CNS inflammation. CNS-infiltrating T cells mostly containing T_H17 cells initiates inflammation in the brain parenchyma whereas the presence of T_H1 cells directed the inflammation towards the spinal cord.

After adoptive transfer, we observed the development of EAE and at different time points we analyzed the infiltrating cell populations from the CNS. The most potent inducer of EAE was the T cell population cultured under conditions, which favored the production of GM-CSF. These animals showed a significantly earlier disease onset and higher clinical score compared to the other groups. Animals receiving T_H17 cells developed EAE but with a more moderate progression

and the onset of disease was considerably delayed compared to the previous experimental group.

T_H1 cells were able to induce tissue destruction in the CNS but among the three groups, these cells had the weakest encephalitogenic potential, which is apparent from the very late commencement of disease and the mildest clinical score with a fast recovery. Interestingly, this finding contradicts several publications, which showed that *in vitro* generated T_H1 cells are able to cause EAE and their pathogenicity is as potent as of T_H17 cells. We cannot provide a straightforward explanation to the phenomena although the observed effect could be due to either the use of animals with different background or the difference in cell culture conditions (Chitnis et al., 2001; Haak et al., 2009b; Yang et al., 2009).

A previous study indicated that *in vitro* generated T_H17 cells are impaired in their ability to transfer disease to susceptible recipients if IL-23 is not present in the culture medium. The observed effect is most likely attributed to the IL-10 blocking capacity of IL-23. TGF-beta and IL-6 induce the expression of IL-10, which is a known effector cytokine of regulatory T cells, thereby ameliorating EAE development (Bettelli et al., 1998; McGeachy et al., 2007). Our findings partially contradicted to this study, hence blockade of IL-23 did not reduce the pathogenic potential of *in vitro* generated T cells in an adoptive transfer experiment. Furthermore, this observation indicated that the presence of IL-23 is not substantial for the generation of pathogenic T helper cells *in vitro*.

After adoptive transfer, we observed that animals receiving GM-CSF-expressing cells developed EAE with the earliest onset, the highest disease incidence and the most exacerbated clinical signs when compared to the other two groups. However, this is not surprising because several publications affirm this observation. It is well known that the absence of GM-CSF completely blocks EAE development in susceptible rodents although the mechanism is completely elusive (Hamilton, 2008b; Ponomarev et al., 2007). Administration of rGM-CSF restores EAE susceptibility and in wild-type animals additional rGM-CSF exacerbates disease development (McQualter et al., 2001). It was shown by Marusic and her colleagues that in a T cell receptor transgenic animal (MBP) transfection of T cells by GM-CSF expressing retrovirus drastically enhances EAE. Interestingly, they were unable to detect GM-CSF expression in the CNS under inflammatory conditions by RT-PCR when they just used a GFP-expressing control virus (Marusic et al., 2002). This is contradicting to our and others observations although we cannot exclude the impact of the influence of a different system although this seems to be insufficient explanation for the observed phenomena.

Stability of different T cell subsets

T_H1 cells are considered to be a fairly stable cell subset while there is an ongoing debate about the stability of the T_H17 cell population. It is thought that T_H17 cells do not represent a terminally differentiated subset and under certain conditions they can acquire characteristic properties of regulatory cells (Afzali et al.; Locksley, 2009; Zhou et al., 2009b). After analyzing the cytokine secretion pattern of invading cells from the CNS, we concluded that in all groups invading T cells expressed robust amounts of GM-CSF although prior to transfer only cells cultured under GM-CSF skewing conditions secreted high amounts of this protein. T_H1 cells in parallel to IFN-γ secretion started producing GM-CSF although the dominant cytokine remained IFN-γ. T_H17 cells expressed high levels of GM-CSF and IFN-γ when they were isolated from the CNS at peak disease, however they maintained high production of their hallmark cytokine, IL-17A. T cells, producing only GM-CSF prior to transfer, initiated a genetic program, which at the end resulted in IFN-γ and IL-17A production. Taking into account all the data, it gave the impression that GM-CSF production is in direct proportion with disease severity and it is needed for encephalitogenicity.

Despite the initial cytokine secretion differences, T_H cells could switch polarization pattern *in vivo* and acquire the ability of producing those cytokines, which are important mediators of inflammation (Bending et al., 2009). However, this is not unexpected at all, since the *in vivo* system contains all factors required for IL-17 or IFN-γ production. In the inflamed CNS, IL-12 is highly up regulated, which induces the expression of IFN-γ while presence of other factors (IL-6, TGF-beta) might contribute to the initiation of IL-17 production (Korn et al., 2009). Furthermore, *in vivo* is completely unknown which factor might initiate the production of GM-CSF although IL-23 is one of the most probable candidates.

Interestingly, cells generated under conditions, which impaired the production of IFN-γ, initiated in some cases atypical disease development. Atypical EAE is characterized by the loss of orientation, constantly spinning tail and tiff body texture. These clinical signs of EAE have been already observed and described in animals, which lack either IFN-γ or its receptor. Most likely this phenotype is related to the penetration ability of cells into the CNS. It has been shown that T_H17 cells preferentially infiltrate the brain parenchyma while T_H1 cells invade the spinal cord. (Cox et al., 2008; Janke et al.; Stromnes et al., 2008)

Our described experimental setting enabled us to study the effect of certain polarizing conditions on T cell behavior during EAE. However, the injected cell population was not completely avoid of cells producing the other cytokines. Thereby we could not exclude completely the contribution of IFN-γ and IL-17 to the initiation of autoimmunity. In order to maximize the purity of our T cell pool

we applied a cell surface cytokine capture (CSCC) assay, which permitted us to specifically select T cells producing only GM-CSF, IFN-γ or IL-17. This method elegantly allows the enrichment of any cell population expressing a certain type of cytokine. In contrast to intracellular cytokine staining, the CSCC assay leaves the investigated cell population intact and it can be used for further i*n vivo* or *in vitro* manipulations. All the sorted, pure T cell populations were viable and were able to induce disease and interestingly by the use of this method, we could drastically reduce the number of cells needed for disease initiation.

After adoptive transfer, we could observe similar EAE development to the previous experiment in which GM-CSF secreting cells were the most pathogenic, followed by IL-17 producing cells while T_H1 cells were mildly pathogenic based on the clinical scoring. GM-CSF secretion seemed to be required for early disease onset and for the maintenance of permanent paralysis. In contrast, cells producing mainly IL-17 or IFN-γ caused a delayed disease onset and the severity of EAE remained moderate. Even more, animals receiving IFN-γ expressing cells showed faster recovery and the incidence were lower compared to the other two groups.

However, this is not surprising because several publications indicated already that IFN-γ might exert protective effects during autoimmunity. Local but not systemic administration of IFN-γ results in complete suppression of clinical signs but in contrast, blockade of IFN-γ prior to onset engraves the progression of EAE (Ferber et al., 1996; Krakowski and Owens, 1996; Leonard et al., 1995). IFN-$\gamma^{-/-}$ animals are more susceptible to EAE and we speculate that the production of GM-CSF is not suppressed anymore by the presence of IFN-γ, which at the end results in the observed phenotype. Our *in vitro* observations supported our hypothesis that IFN-γ *in vivo* and *in vitro* functions as a negative regulator of GM-CSF production thereby interfering with EAE development.

GM-CSF secreted by invading T cell is essential for EAE development

To ultimately prove that T cell derived GM-CSF in the CNS is fundamental for the initiation of EAE, we took advantage of the use of different single cytokine deficient cells. As it has been demonstrated IL-17A and IFN-γ are not fundamental for the induction of autoimmunity in the CNS and as we expected cells lacking these cytokines readily invaded the CNS and caused ascending paralysis in the recipient animals. As we observed previously, the lack of IFN-γ modified the infiltration pattern of cells and some of these animals developed atypical EAE, making the scoring incomparable to the other groups. Cells not being able to express IL-17A invaded the CNS and

produced robust amounts of GM-CSF, once again consolidating the earlier observations that IL-17A is dispensable for EAE development. Its production closely correlates with the level of inflammation but it appears that its activity is not required to induce or maintain local inflammation in the brain.

GM-CSF deficient T cells were unable to infiltrate the CNS and commence an inflammatory cascade event, which ultimately leads to leukocyte invasion in the CNS. However, the pro-inflammatory cytokine production of GM-CSF$^{-/-}$ cells is not affected by the lack of GM-CSF and the EAE resistance most likely does not originate from a T cell intrinsic defect. *In vitro* stimulated T cells produce comparable amounts of IL-17 and IFN-γ to wild type T cells showing that GM-CSF deficiency does not impair the production of some of the known T_H1, T_H17 cytokines.

Previous publications showed that despite the fact that GM-CSF is one of the most important growth factors for leukocyte differentiation, its lack does not cause a systemic defect on hematopoiesis. The only so far described side effect of its genetic loss can be observed in the lungs where alveolar macrophages are negatively affected. This defect can be partially compensated by the addition of rGM-CSF (Carey and Trapnell).

Despite their IFN-γ and IL-17 producing ability, GM-CSF deficient cells failed to invade the CNS and cause paralysis. The exact mechanism by which GM-CSF contributes to the development of autoimmunity is not yet properly understood. By being an important factor for maturation of antigen presenting cells, it is feasible that its lack interferes with antigen presentation locally. Activated T cells should re-encounter their cognate antigen in the target organ to efficiently induce the tissue destruction and the recruitment of other inflammatory cells. The lack of GM-CSF might down-regulate the expression of MHC II on the surface of local DCs, or the expression of other co-stimulatory molecules could be affected thereby hampering the formation of the immune synapse between T cells and DCs (Xiao et al., 2007). Another possibility is that the missing GM-CSF negatively influences the priming process of T cells although so far there is no direct evidence to this hypothesis. It has been shown that in a GM-CSF deprived environment DCs fail to produce sufficient amount of IL-6, which is essential for T cell survival. T cells without satisfactory IL-6 signaling initiate an apoptotic program that ultimately leads to their death. Although in our experiment T cells were primed in a GM-CSF deficient animal but later on, after *in vitro* restimulation with their cognate antigen MOG_{35-55} in the presence of IL-23, they were transferred into a GM-CSF sufficient animal. After adoptive transfer, the already primed T cells were exposed to proper level of IL-6, which most likely was sufficient to maintain their survival. However, we cannot exclude the possibility that IL-6 plays a central role during priming and its presence cannot restore pathogenicity of already primed T cells (Sonderegger et al., 2008a).

GM-CSF secretion requires the presence of Rorγt and is inhibited by the presence of IL-27

After evaluating the impact of different culture conditions on pathogenic properties of single cytokine deficient cells, we wanted to know whether GM-CSF production depends on the action of a known transcription factor. Several publications indicated the pivotal role of T-bet and Rorγt in the development of T_H1 and T_H17 cells, respectively (Ivanov et al., 2006; Ivanov et al., 2007; Szabo et al., 2000). It was shown that both T-bet and Rorγt-deficient animals are EAE resistant although because of a different reasons. Interestingly, T-bet deficiency, in contrast to the other T_H1 hallmark genes, results in complete EAE resistance while the deletion of IFN-γ, IFN-γR or STAT5 results in increased disease severity. Loss of T-bet influences not just T_H1 development but it has a negative impact on the ability of DCs to prime T cells (Mathur et al., 2006; Nath et al., 2006; Wang et al., 2006).

Rorγt, which has been demonstrated to be the main transcription factor for T_H17 generation, not just only influences T_H17 polarization but it is also essential for the formation of lymph nodes. It is likely that EAE resistance is due to the combined effect of the two previously mentioned defects. We analyzed the expression pattern of these transcription factors in order to shed light onto the molecular mechanisms governing the production of GM-CSF. As it is expected under T_H1 polarizing conditions, IFN-γ-producing cells expressed high levels of T-bet and we could not detect any expression of Rorγt.

Administration of T_H17 polarizing factors induced the expression of Rorγt in activated T cells and almost all IL-17 producing cells were positive for Rorγt. Interestingly, a huge population of IL-17 negative cells expressed Rorγt and this fact might suggest that Rorγt and IL-17 are not tightly interconnected and IL-17 expression is transient by T cells. Furthermore, GM-CSF-expressing cells were positive for Rorγt expression as well. T-bet was absent from both, T_H17 and GM-CSF-expressing cells, indicating its exclusive role only in T_H1 polarization. The fact that GM-CSF-secreting T cells expressed Rorγt suggested the notion that the secretion of this cytokine is dependent on the action of this T_H17 hallmark transcription factor.

To further characterize the necessity of Rorγt for GM-CSF production we took advantage of mice, which carried a mutated allele of the Rorc gene. Stimulating Rorc-deficient splenocytes under different conditions we observed that IFN-γ expression is not hampered but the secretion of IL-17A as well of GM-CSF is highly reduced in the absence of this transcription factor. This fact nicely correlated with our previous observations and suggested that the absence of GM-CSF is the factor responsible for the EAE resistance of $Rorc^{-/-}$ mice, while the absence of IL-17 could be a secondary effect due to the lack of inflammation.

Recently it has been described that another IL-6 family member is involved in the regulation of autoimmunity, namely IL-27. It has been demonstrated that IL-27 deficiency results in exacerbated EAE development and this fact suggested that IL-27 behaves as anti-inflammatory cytokine during EAE. It exerts its effect by enhancing the action of T-bet and subsequently increasing the susceptibility of T cells to IL-12 stimulation. As previously described, T-bet is a negative regulator of T_H17 polarization. The inhibitory effect of IL-27 on T_H17 has been well characterized and the EAE exacerbating effect of the lack of IL-27 expression is thought to originate from its suppressor capacity on T_H17 development (Batten et al., 2006; Diveu et al., 2009; Fitzgerald et al., 2007b; Stumhofer et al., 2006).

Administration of rIL-27 already at low concentration efficiently blocks IL-17 expression under T_H17 polarizing condition. Interestingly, not just IL-17 expression is reduced but by the addition of rIL-27 but the secretion of GM-CSF can be inhibited completely *in vitro*. Again, our results indicated that an effect previously ascribed to be mediated though the blockage of T_H17 cells is rather mediated by through the inhibition of GM-CSF secretion.

Conclusions

IL-23 is an essential factor for EAE development but until recently, the participation in T_H17 cell generation was its major known function. We demonstrated that *in vivo* IL-23 plays a crucial role in the migration of T cells and in its absence MOG-specific T cells fail to leave secondary lymphoid structures and traffic to the CNS. Furthermore, the loss of IL-23 engagement impairs the production of pro-inflammatory cytokines such as IL-17A and GM-CSF. We revealed that GM-CSF production is inhibited by IFN-γ, IL-12 and IL-27 and depends on the action of Rorγt. GM-CSF-expressing cells can be generated *in vitro* and they are more potent inducers of EAE than T_H17 or T_H1 cells. Taken together, GM-CSF rather than IL-17A or IFN-γ is the encephalitogenic factor produced by T_H cells which resolves and integrates virtually all conflicting results regarding the function of RORc, IL-12, IFN-γ, IL-23 and IL-27 mutants and their phenotype during EAE.

Material and methods

Enzymes

Restriction enzymes

Restriction endonucleases cut double stranded DNA at specific palindromic recognition sites. All endonucleases used in our laboratory cut DNA within the target sequence, creating 3' or 5' overhanging ends, the so-called sticky ends.

All following restriction enzymes were purchased from New England Biolabs (USA). Restriction enzymes utilized are:

*BamH*I	100 U/ µl
*Bgl*I	10 U/ µl
*Cla*I	5 U/ µl
*EcoR*I	20 U/ µl
*EcoR*V	20 U/ µl
*Hind*III	50 U/ µl
*Pvu*II	10 U/ µl
*Spe*I	100 U/ µl
*Xho*I	20 U/ µl

Polymerases

A polymerase synthesizes DNA complementary to template DNA. It starts from ssDNA primers bound to the complementary part on the template strand. The most frequently used polymerase is the *Taq* polymerase, which was cloned from the thermophilic bacterium *Thermus aquaticus.* It is active over a broad temperature range, thus making it suitable for polymerase chain reaction cycling.

Since the *Taq* polymerase does not show any proofreading activitiy, it was combined with *Pfu* polymerase from *Pyrococcus furiosus* for cloning purposes. The *Pfu* polymerase exhibits an intrinsic 3'-5' exonuclease proofreading activity and therefore substantially decreases the mutation rate of the PCR.

Three different polymerases were used:

• commercial *Taq* polymerase purchased from NEB(USA)(5U/µl)

• commercial *HiFI* polymerase purchased from Invitrogen (USA)(2.5U/µl)

T4 DNA Ligase

T4 DNA ligase (New England Biolabs, USA, 400U/µl) from bacteriophage T4 ligates over hanging or blunt ends of double stranded DNA and was used for cloning of the different constructs.

Shrimp Alkaline Phosphatase (SAP)

This phosphatase (New England Biolabs, 1U/µl) is used to dephosphorylate 5' DNA fragments. For cloning procedures, usually the digested vector was dephosphorylated to decrease re-ligation efficiency. Since the fragment that is supposed to be ligated into the vector still carries its phosphate groups ligation occurs between vector and insert and not between the vector ends themselves.

Plasmids

pGEM-T Easy plasmid

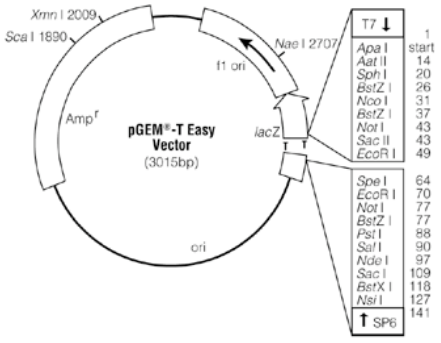


Figure 11. Schematic map of the pGEMT Easy Vector (taken from the Promega manual)

The map shows the multiple cloning site, the ampicillin resistance cassette and the lacZ gene. The pGEM-T Easy Vector System is aconvenient system to subclone PCR products prior to sequencing or further cloning steps. The vectors are prepared by cutting Promega's pGEM-T Easy Vector with *Eco*RV and adding a 3´ terminal thymidine to both ends. These single 3´-Toverhangs at the insertion site greatly improve the efficiency of ligation of a PCR product into the plasmids by preventing recircularization of the vector and providing a compatible overhang for PCR products generated by certain thermostable polymerases, such as *Taq* polymerase. These polymerases add a single deoxyadenosine to the 3´-ends of the amplified fragments in a template-independent fashion.

pRapidflirt

It comprises of loxP sites, flp sites, Neo and Ampicillin resistance ORF and HSV-tk gene.

Bacteria

Competent bacteria

Chemically competent bacteria for plasmid transfection were generated from two different *Escherichia coli* (*E. coli*) strains:

DH5α	F-, endA1, hsdR17, (r-,m+k), supE44, th*i*-1, 1-, recA1, gyrA96, relA1, argF-, lac zya U169, O80lacZ M15
TOP10 (Invitrogen,)	F-, mcrA, (mrr-hsdRMS-mcrBC), lacX74, deoR, recA1, araD139, (ara-leu)7697, galU, galK, rpsL (StrR), endA1, nupG, O80lacZ M15

Molecular biology techniques

Preparation of plasmid DNA

Plasmid DNA preparation from *E. coli* cells was performed using alkaline lysis followed by denaturation of proteins and precipitation of chromosomal DNA.

Bacteria are lysed by treatment with a solution containing sodium dodecyl sulfate (SDS)(denatures bacterial protein) and NaOH (denatures chromosomal and plasmid DNA).

The mixture is neutralized with potassium acetate, causing the covalently closed plasmid DNA to reanneal rapidly. Chromosomal DNA and bacterial proteins are precipitated and removed by centrifugation. The plasmid DNA from the supernatant is then concentrated by isopropanol precipitation.

Resuspension buffer (P1) Lysis buffer (P2)
6.06 g Tris-base 8g NaOH in 950 ml H2O
3.72 g sodium EDTA add 50 ml of 20% (w/v) SDS
solubilize in 800 ml H2O
adjust pH to 8.0
add H2O to 1000 ml final volume
add 100 µg/ml RNase A

Neutralization buffer (P3) TE (Tris/EDTA) buffer
294.5 g potassium acetate 10 mM Tris/HCl pH 8.0
solubilize in 500 ml H2O 1 mM EDTA
adjust pH with acetic acid to 5.5
add H2O to a final volume of 1000 ml

Mini preparation of plasmid DNA

Plasmid DNA was prepared from an overnight culture (5 ml LB medium inoculated with a single *E. coli* colony). Three ml of this culture were used for every lysis. Cells were centrifuged in a microcentrifuge at 13000 rpm for 5 min. The supernatant was discarded and the pellet resuspended in 150 µl of P1. 150 µl of P2 were added. The solutions were mixed by inverting the tube several times. Addition of 300 µl of P3 resulted in precipitation of proteins and chromosomal DNA, which were sedimented after 20 min of centrifugation at 13000 rpm.
The supernatant was transferred into a fresh 1.5 ml tube. Plasmid DNA was precipitated with isopropanol, washed with 70 % (v/v) ethanol and resuspended in TE buffer.

Maxi preparation of plasmid DNA

Large quantities of plasmid DNA were isolated with the Plasmid Maxi Kit (QIAGEN, Hilden) 100-200 ml of an overnight *E. coli* culture were used for every lysis. The DNA was purified using anion exchange chromatography. The preparations were carried out according to the manufacturer's instructions.

DNA extraction from mouse tail biopsies

0.5 ml of tail lysis buffer was added to a freshly cut or frozen tail biopsy (0.5 cm). Digestion of proteins was performed overnight at 56°C by proteinase K treatment. Undigested organic

material was removed by centrifugation (13000 rpm, 10 min). The supernatant was transferred to fresh tubes. Genomic DNA was precipitated with 0.5 ml isopropanol. The DNA was sedimented for 10 min at 13000 rpm and washed with 70% (v/v) ethanol and subsequently was dried and then dissolved in 200 µl TE buffer at 56°C for approximately 2 h. It was stored at 4°C.

Tail lysis buffer
100 mM Tris/HCl pH 7.5
5 mM EDTA
0.2% (w/v) SDS
200 mM NaCl
100 µg/ml proteinase K (added freshly prior to lysis)

DNA extraction from ES cells (96-well microtiter plates)

After washing ES cells twice with 100 µl PBS, 50 µl of ES cell lysis buffer was added to each well. The plates were transferred into a pre-warmed humidified box in which digestion of cellular proteins proceeded overnight at 56°C. After lysis and denaturation, samples were cooled to room temperature for 1 h. ES cell derived genomic DNA was precipitated with absolute ethanol (100 µl per well) for an additional hour at room temperature. DNA strands were visible under the microscope. To remove the ethanol the plates were inverted and the wells carefully drained on paper towels. DNA usually remained attached to the plastic surface. Every well was washed three times with 150 µl of 70 % (v/v) ethanol per well. DNA was air-dried and ready for further processing.

PBS (phosphate buffered saline) ES cell lysis buffer
137 mM NaCl 10 mM Tri/HCl pH 7.5
2.7 mM KCl 10 mM EDTA
4.3 mM Na2HPO4
.7H2O 0.5 % (w/v) sarcosyl (sodium lauryl sarcosinate)
1.4 mM KH2PO4 10 mM NaCl
0.4 mg/ml proteinase K (added prior to lysis)

Phenol/Chloroform extraction of DNA

In order to remove protein contamination from an aqueous DNA solution, a phenol/chloroform extraction was performed (Kirby, 1957; Palmiter, 1974). Phenol and chloroform efficiently

denature proteins. Chloroform has an important role in stabilizing the boundary between the aqueous phase and the phenol phase.
One volume of a phenol/chloroform-mixture (1:1) was added to an aqueous DNA solution. The sample was vortexed for at least one minute and then centrifuged for 3 min at 13000 rpm. The aqueous phase was transferred into a fresh tube. Residual phenol was removed by washing two times with one volume of chloroform (13000 rpm, 2 min). The purified aqueous phase was transferred into a new tube, and the DNA was ethanol precipitated.
In case of phenol/chloroform extraction of linearized targeting vector prior to transfection, the precipitated DNA was kept in 70 % (v/v) ethanol, stored at 4°C to guarantee sterility for cell culture work. Prior to the transfection, the DNA was dried and dissolved in transfection buffer under a sterile hood.

Isolation of DNA fragments from agarose gel

The isolation of desired DNA fragments after restriction digest or PCR was performed using gel electrophoresis. Depending on the size of the DNA fragment, the DNA was separated on a 0.7–2.5 % (w/v) agarose gel.
The respective bands were identified with a UV light source (λ=366 nm) and cut out with a sterile scalpel. Elution of the DNA was carried out using the QIAquick Gel Extraction Kit (QIAGEN, Hilden) following the manufacturer's instructions. Fragments larger than 8 kb were isolated using the QIAEX II Gel Extraction Kit (QIAGEN, Hilden) following the suggested protocol.
The principle of both methods is based on anionic exchange chromatography after acidic solubilization of the gel.

Restriction digest of plasmid DNA

Restriction digests of plasmids were carried out as suggested by the manufacturer.
In general, DNA was digested with 1/10 volume restriction enzyme 10 x buffer and 2-10 units of enzyme per µg DNA. The volume of the enzymes never exceeded 10 % of the final restriction digest volume to prevent inhibition of the reaction by glycerol which is part of the
The digests were incubated at the recommended temperatures (usually 37°C) for 1-12 hrs.

Restriction digest of ES cell derived genomic DNA

Genomic DNA from murine ES cells on 96 well plates was isolated according to the previously described protocol The air-dried DNA was digested in a total volume of 35 µl. To allow efficient

digest in the presence of proteins and RNA, spermidine (1 mM), BSA (100µg/ml), DTT (1mM) and RNaseA (50µg/ml) were added to the restriction mix. 1-2 U/µl of restriction enzyme per well were used to ensure thorough digestion of the genomic DNA.

Southern blot

Southern blotting is the transfer of DNA fragments from an electrophoresis gel to a membrane. The transfer results in immobilization of the DNA fragments, so the membrane carries a semipermanent reproduction of the banding pattern of the gel. After immobilization, the DNA can be hybridized with radioactively labeled probes and visualized by autoradiography (Southern, 1975).

Genomic DNA was digested with restriction endonucleases, and the resulting fragments were separated according to size by agarose gel electrophoresis on a 0.7 % (w/v) agarose gel. The DNA was denatured *in situ* and transferred onto a positively charged nylon membrane.

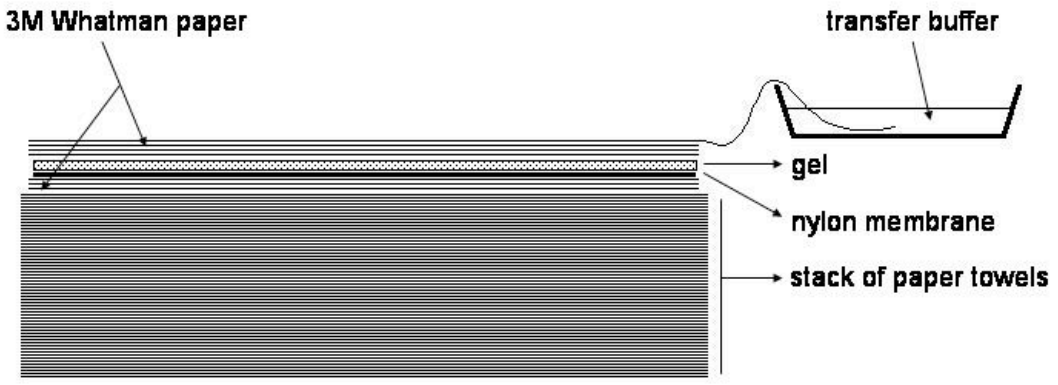
Blotted DNA was fixed by incubation at 65°C for 1h. The DNA was hybridized to a P^{32} radioactively labeled DNA probe specific for the desired genomic region. Autoradiography was used to locate the positions of bands to which the probe hybridized.

Preparation and transfer of DNA samples

Southern blot analysis was used to identify the targeted allele in the genome of ES cells and for typing of genetically modified mice.

The digested DNA and a DNA size marker were separated on a 0.7 % agarose gel overnight (40V, 16 h). The gel was photographed under UV light with a ruler aligned to the 1 kb DNA size marker and its bands were marked with a yellow tip. Afterwards the gel was gently shaken in 0.25 M HCl for 15-20 min, which leads to partial depurination of the DNA, which in turn leads to strand cleavage. The HCl was exchanged with a 0.4 M NaOH solution, which functions as a denaturation agent, to obtain single-stranded DNA. The DNA is ready to be transferred onto a positively charged nylon membrane (Hybond N+, Amersham Biosciences,
UK). The DNA was transferred using downward capillary transfer with an alkaline transfer buffer. Transfer was performed for 4-16 h.

Figure 12. Schematic representation of Southern blot



After completion of the transfer, the membrane was marked with a pencil corresponding to the previously marked size marker. Afterwards the membrane was baked for 1 h at 65°C to immobilize the DNA on the membrane.

Depurination buffer

0.25 M HCl

Denaturation buffer

0.4 M NaOH

Transfer buffer

0.4 M NaOH

0.6 M NaCl

Hybridization

After baking of the membrane, it was moistened with 2 x SSC and thereafter incubated in a rotating oven for at least 2 h at 65°C with prehybridization solution. In the meantime, the DNA probe was labeled with P^{32}. 30-100 ng of DNA (probe) were mixed with 2 µl of random primers (TaKaRa labeling kit) and filled up with water to a final volume of 10 µl. The solution was boiled for 3 min, to obtain single stranded DNA. After a 5 min incubation on ice, 2.5 µl of Bca buffer, 2.5 µl of dNTPs, 6.5 µl of H2O, 1.0 µl of Bca BEST polymerase (all components part of the TaKaRa labeling kit) and 25 µCi 32Pα-CTP were added. The mix was incubated at 50°C for 25 min. The labeling reaction was stopped by adding 100 µl of H_2O. The labeled probe was purified from non-incorporated nucleotides on a Micro Spin S 200 HR column (Amersham Bioscience, UK). The purified probe was boiled for 3 min, incubated on ice for 5 min and finally added to the prehybridization solution. Hybridization was performed by rotating the membrane in the

hybridization solution overnight at 65°C.

To avoid background labeling, the membrane was washed the next day with 2 x SSC until the counts decreased to 100-150 cpm. Afterwards, the membrane was sealed in a plastic bag. The blot was analyzed by phosphoimaging. The sizes of the bands were estimated using their electrophoretic mobility relative to the previously photographed gel or per overlay with the marked bands of the size ladder on the blot.

Prehybridization solution 20 x SSC

1 M NaCl

50 mM Tris pH 7.5

10 % (w/v) dextrane sulfate

1 % (w/v) SDS

250 µg/ml salmon sperm DNA (sonicated)

20 x SSC

3 M NaCl

300 mM sodium citrate pH 7.0

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